THE AUSTRALIAN JOURNAL OF EXPERIMENTAL BIOLOGY AND MEDICAL SCIENCE

Sustained since 1926 by the Endowment of Sir Joseph Cooke Verco for the publication of Medical Research

EDITOR-IN-CHIEF:

MARK L. MITCHELL (ADELAIDE)

CO-EDITORS:

J. B. CLELAND (ADELAIDE), SIR STANTON HICKS (ADELAIDE)

Assisted in the selection of papers for publication by the following

BOARD OF EDITORS:

W. E. AGAR (Melbourne); E. ASHBY (Sydney); L. B. BULL (Melbourne); W. J. DAKIN (Sydney); H. W. DAVIES (Sydney); F. GOLDBY (Adelaide); E. WESTON HURST (Adelaide); T. HARVEY JOHNSTON (Adelaide); C. H. KELLAWAY (Melbourne); J. A. PRESCOTT (Adelaide); W. RAY (Adelaide); A. E. V. RICHARDSON (Melbourne); C. W. STUMP (Sydney); HARVEY SUTTON (Sydney); J. S. TURNER (Melbourne); H. J. WILKINSON (Queensland); J. G. WOOD (Adelaide); H. A. WOODRUFF (Melbourne); W. J. YOUNG (Melbourne).

VOL. XIX.

PUBLISHED BY THE UNIVERSITY OF ADELAIDE

MANAGED FOR THE UNIVERSITY BY THE MEDICAL SCIENCES CLUB OF SOUTH AUSTRALIA

PRINTED AT THE HASSELL PRESS, ADELAIDE

1941

CONTENTS

PART 1. MARCH, 1941

H. F. HOLDEN: "The Ultra-Violet Absorption Spectra of Some Metallo-Porphyrins and of Their Compounds with Globin" 1

R. CHRISTIE AND J. J. GRAYDON: "Observations on Staphylococcal Haemolysins and Staphylococcal Lipase" 9

C. H. KELLAWAY AND E. R. TRETHEWIE: "Tissue Injury by the Toxin of *Cl. Welchii*, Type A" 17

E. C. SLATER: "The Thiochrome Method of Estimating Thiamin (Vitamin B_1) in Urine, Milk and Cereal Products" 29

G. V. RUDD AND F. M. BURNET: "Intranasal Infection of Mice with the Virus of Psittacosis" 33

F. M. BURNET: "Influenza Virus Infections of the Chick Embryo by the Amniotic Route. 2. Titrations and Serum Neutralization Tests" 39

NANCY ATKINSON: "A Study of Some Australian Strains of *Erysipelothrix*" 45

E. V. KEOGH, R. T. SIMMONS AND HAROLD WILSON: "*Streptococcus Pyogenes* (Group A): A Review of 1,341 Strains Collected from Various Human Sources" 51

A. E. PLATT: "Observations on the Rôle of Sodium Chloride in the Agglutination of Bacteria by Antibody" 57

A. H. ENNOR AND CHARLOTTE M. ANDERSON: "The Influence of Anterior Pituitary Extracts upon the Rate of Oxidation and Reduction of Glutathione in Tissues" 69

D. J. W. SMITH: "Studies in the Epidemiology of Q Fever. 7. The Biology of *Haemaphysalis Humerosa* Warburton and Nuttal (Acarina, Ixodidae) in Queensland 73

PART 2. JUNE, 1941

C. H. KELLAWAY AND E. R. TRETHEWIE: "The Injury of Tissue Cells and the Liberation of Pharmacologically Active Substances by the Toxins of *Cl. Welchii* Types B and C" 77

H. F. HOLDEN: "The Cobalt Compounds of Protoporphyrin" .. 89

BARBARA COOKE AND RUPERT J. BEST: "The Inactivating Effect of Salicylate on Suspensions of Some Animal Viruses. (Herpes, Infectious Myxoma, Shope Fibroma, Vaccinia, Poliomyelitis)" .. 93

F. M. BURNET AND M. FOLEY: "Influenza Virus Infections of the Chick Embryo by the Amniotic Route. 3. Changes in the Activity of Influenza Virus on Continued Amniotic Passage" 101

F. S. COTTON: "Studies in Respiration. 4. The Effect of Varying the Time of Apnoea on the Occurrence and Extent of the Oxygen Trough of Respiration" 107

CHARLES SWAN: "The Route of Transmission of the Virus of Infectious Myxomatosis of Rabbits to the Central Nervous System" .. 113

P. FANTL AND CHARLOTTE M. ANDERSON: "The Breakdown of Hexose Monophosphates in Liver Pulp" 117

E. SINGER: "Experiments on the Survival of Rickettsiae in Cell-Free Media" 123

E. SINGER: "Studies on the Hormonal Antigens of the Anterior Pituitary Gland" 125

D. J. W. SMITH: "Studies in the Epidemiology of Q Fever. 8. The Transmission of Q Fever by the Tick *Rhipicephalus Sanguineus*" .. 133

WILLIAM ARTHUR RAWLINSON: "The Effect of Oxidizing Agents on Haemocyanin" 137

CHARLES SWAN: "Experimental Poliomyelitis. 1. The Effect of Faradic Stimulation on the Localization of Paralysis Induced by the Intranasal Inoculation of Poliomyelitis Virus" 143

PAGE

A. B. BECK: "Studies on the Copper Content of the Milk of Sheep and of Cows" 145

F. H. SHAW: "The Action of Alkali on the Chemical and Physiological Properties of Adrenaline" 151

E. A. HANSON: "A Note on the Metabolism of Chloroplast Protein" .. 157

A. W. TURNER AND CATHARINE E. EALES: "An Aerobic Sporulating Bacillus that Forms Rotating and Migrating Colonies" .. 161

PART 3. SEPTEMBER, 1941

A. W. TURNER AND CATHERINE E. EALES: "Motile Daughter Colonies in the *Cl. Oedematiens* Group and Some Other Clostridia (*Cl. Botulinum C, Cl. Tetani, Cl. Septicum*)" 167

E. R. TRETHEWIE: "The Liberation of Pharmacologically Active Substances from Tissues by Chloroform" 175

SYDNEY D. RUBBO, MARGARET MAXWELL, R. A. FAIRBRIDGE, AND J. M. GILLESPIE: "The Bacteriology, Growth Factor Requirements and Fermentation Reactions of *Clostridium Acetobutylicum* (Weizmann)" 185

G. REID: "The Reaction of Muscle to Denervation in Cold Blooded Animals" 199

IAN ROBERTSON AND F. H. SHAW: "A Contribution to the Pharmacology of Adenyl Compounds, with a Note on Their Occurrence in Red Blood Cells" 207

ALFRED GOTTSCHALK: "The Mechanism of the Pasteur Effect in Alcoholic Fermentation by Yeast Cells" 211

E. A. HANSON, B. S. BARRIEN AND J. G. WOOD: "Relations between Protein-Nitrogen, Protein-Sulphur and Chlorophyll in Leaves of Sudan Grass" 231

F. M. BURNET AND M. FOLEY: "Infections of the Respiratory Tract of Chick Embryos by the Viruses of Psittacosis and Infectious Laryngotracheitis. A Comparison with Influenza Virus Infections" .. 235

A. B. CORKILL AND J. F. NELSON: "The Influence of Adrenal Cortical and Sex Hormones on Liver Glycogen" 241

R. A. WYNDHAM: "Experimental Demyelination of the Central Nervous System. 2. Respiratory Enzyme Systems of the Brain in Poisoning with Cyanide and with Azide" 243

PART 4. DECEMBER, 1941.

A. B. BECK: "Studies on the Blood Copper of Sheep and of Cows" .. 249

STEPHEN WILLIAMS: "The Occurrence of Lipoid Structures in Cultures of *Streptobacillus Moniliformis*" 255

A. R. ROBINSON: "Technique of Adrenalectomy in the Guinea-Pig and the Maintenance of the Adrenalectomized Animals" 261

R. N. ROBERTSON: "Studies in the Metabolism of Plant Cells. 1. Accumulation of Chlorides by Plant Cells and its Relation to Respiration" 265

P. FANTL: "A Simple Weight-Burette for Use in Organic Analysis" .. 279

F. M. BURNET: "Influenza Virus Infections of Cynomolgus Monkeys" .. 281

F. M. BURNET: "Growth of Influenza Virus in the Allantoic Cavity of the Chick Embryo" 291

C. H. KELLAWAY, G. REID AND E. R. TRETHEWIE: "Circulatory and Other Effects of the Toxin of *Cl. Septique*" 297

GEORGE HUMPHREY: "The Determination of Glycogen in Oysters" .. 311

J. G. WOOD: "Relations Between Respiration Rate and Metabolism of Carbohydrate, Protein and Organic Acids in Leaves" 313

R. CHRISTIE AND E. A. NORTH: "Observations on Staphylococcal Beta Toxin and its Production by Strains Isolated from Staphylococcal Lesions in Man" 323

R. CHRISTIE AND HAROLD WILSON: "A Test of Staphylococcal Fibrinolysis" 329

THE ULTRA-VIOLET ABSORPTION SPECTRA OF SOME METALLO-PORPHYRINS AND OF THEIR COMPOUNDS WITH GLOBIN

by H. F. HOLDEN[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Accepted for publication 4th October, 1940.)

Some years ago Hill and Holden (1926) prepared globin, the native protein of oxyhaemoglobin, and described its combination with haematin to form methaemoglobin, with porphyrins to form porphyrin-globins and with some metallo-mesoporphyrins. They relied, in the main, on the change in the wave-lengths and appearance of the bands of the visible spectra as indications of combination having occurred. These changes are very marked in the case of protoporphyrin and they attempted to titrate spectrophotometrically a solution of globin with both haematin and protoporphyrin with a view to establishing the stoicheiometric relationship between the latter and globin. The reagents used were, however, too deleterious for the determination to be regarded as very satisfactory. Since then an improved method for the preparation of globin has been described by Anson and Mirsky (1930) and much work has been done on the physical constants of this protein. The spectroscopy of many porphyrins and their metallic complexes has also been investigated, particularly in the visible region of the spectrum. The ultra-violet absorption has been less studied. Holden (1936), using an ultra-violet spectrograph and rotating sector, studied the equilibrium between methaemoglobin, haematin and globin. Later he (1937) measured the ultra-violet absorption spectrum of protoporphyrin in the presence of globin and believed that the compound between them was most stable near the neutral point. Owing to the slow speed of the available instrument a few determinations only could be made in a time considered reasonable on account of the instability of globin and of its rapid photo-decomposition in the presence of porphyrins.

An ultra-violet spectrophotometer incorporating an échelon cell has recently become available, which permits of much more rapid and satisfactory determinations of the absorption spectra of unstable substances. This paper describes measurements made with it on the spectra of some metallo-protoporphyrins in various solvents and in the presence of globin. An attempt was also made to determine the quantitative relationships between haemin, protoporphyrin and copper-protoporphyrin with reference to their power of combining with globin.

Experimental. The globin used was that of the ox prepared by the method of Anson and Mirsky (1930). It is perhaps worth noting that a second extraction of the mass of denatured globin after the removal by filtration of the native globin solution gives a second yield which is frequently less contaminated with pigments and more stable than that first extracted. The globin solution was stored at pH 8 in the refrigerator and filtered immediately before use. Care was exercised to see that no solution whose absorption was to be measured showed any light scattering. The haemin used was recrystallized from pyridine-chloroform-glacial acetic acid. The protoporphyrin and its copper, nickel, cobalt and zinc complexes were prepared as described by Fischer and Putzer (1926). The metallo-porphyrins were recrystallized from pyridine and glacial acetic acid. The addition of water to the suspension of protoporphyrin in pyridine, as described by the German workers, to increase the yield was not found as satisfactory as leaving the suspension of crystals in pyridine in a refrigerator overnight. By this means a better separation of the crystals from the tarry matter was obtained. The latter did not contain enough protoporphyrin to yield crystals of copper-protoporphyrin when an attempt was made to prepare it thus as suggested by Fischer and Putzer (1926).

[1] This work was aided by a grant from the National Health and Medical Research Council.

As a preliminary some determinations were made of the ultra-violet absorption spectrum of haematin in various solvents. Hicks and Holden (1929) for their measurements on acid haematin in alcohol used some haemin crystals which had been extensively washed to remove impurities but which had not been recrystallized. A solution was accordingly prepared from recrystallized haemin in a manner similar to that previously employed. Owing to the shorter light path of the cell used it was, however, considerably more concentrated. It was found that the height of the main band was less than that measured with the presumably less pure material and, further, that the value obtained diminished rapidly on standing. Similar values were obtained with a sample of recrystallized material kindly furnished by Dr. Lemberg. A series of determinations was therefore made on the absorption curve of acid haematin in alcohol with either sodium hydroxide or pyridine to effect the preliminary conversion of haemin to haematin. The concentration of haematin in the solutions actually measured was about 0·015–0·02 mg. per c.c. It was of course accurately measured in each case. The results of these determinations appear in Table 1. Determinations were also made of the ultra-violet absorption spectrum of haematin in pyridine diluted with chloroform and in other solvents e.g. ethyl acetate and cellosolve. These last yielded no information of any interest, the pigment appearing to be entirely in the colloidal state.

TABLE 1.

The maximum absorption of haematin dissolved in alcoholic hydrochloric acid.

A. The haemin was dissolved first in 0·1 N NaOH, 0·2 c.c. per mg. The brackets indicate sucessive determinations on one solution.

Concentration of HCl in gm. equivalent per litre.	Time in minutes between making up the solution and photography.	$\epsilon 10^{-2}$
0·002	5	122
„	120	57
0·002	5	124
„	35	107
„	65	50
„	125	30
0·0012	5	173
0·0008	5	170
„	45	159
„	120	123
0·00025	5	153
0·0022	5	153
0·001	5	160
„	90	90
0·02	5	146
0·01	5	156
0·006	5	156
„	90	75

B. 2·45 mg. haemin first dissolved in 0·2 c.c. pyridine $CHCl_3$ added to 10·0 c.c. 0·6 c.c. of this solution diluted to 5·0 c.c. with 0·1 N alcoholic hydrochloric acid.

0·09	5	133
„	50	133
„	170	133
„	280	124

As Hill and Holden (1926) observed, protoporphyrin, when dissolved in dilute sodium hydroxide solution, does not react with globin. They poured a solution of protoporphyrin dissolved in a mixture of acetic and sulphuric acids into a solution of globin made alkaline with sodium carbonate. In the present work protoporphyrin dissolved in alcoholic hydrochloric acid and in water containing a little pyridine and ammonia has been added to solutions of globin in order to effect the combination. The protoporphyrin compounds of copper, nickel and zinc which are quite insoluble in dilute alkalies dissolve readily in water containing about 6–8 p.c. pyridine and 0·4 p.c. ammonia. So dissolved they readily combine with globin. The solutions for measurements with the Hartridge spectrometer were prepared in the same way as these for ultra-violet spectrophotometry but were somewhat more concentrated.

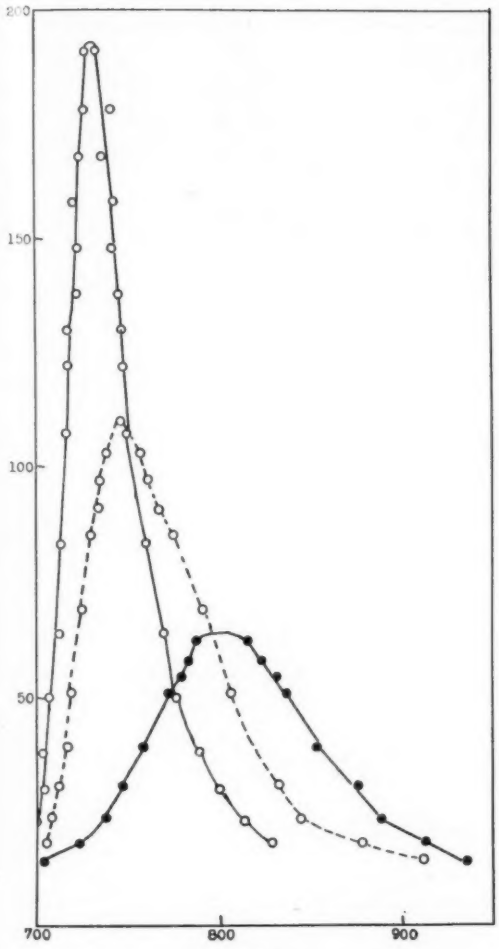


Fig. 1.

—o—o— Protoporphyrin in alcoholic hydrochloric acid.
—•—•— Protoporphyrin in water.
– – o – – o – – Protoporphyrin-globin.

Ordinates represent $\epsilon 10^{-3}$
Abscissae represent $\nu 10^{-12}$

$$\epsilon = \frac{M}{cd} \log \frac{I_0}{I}.$$

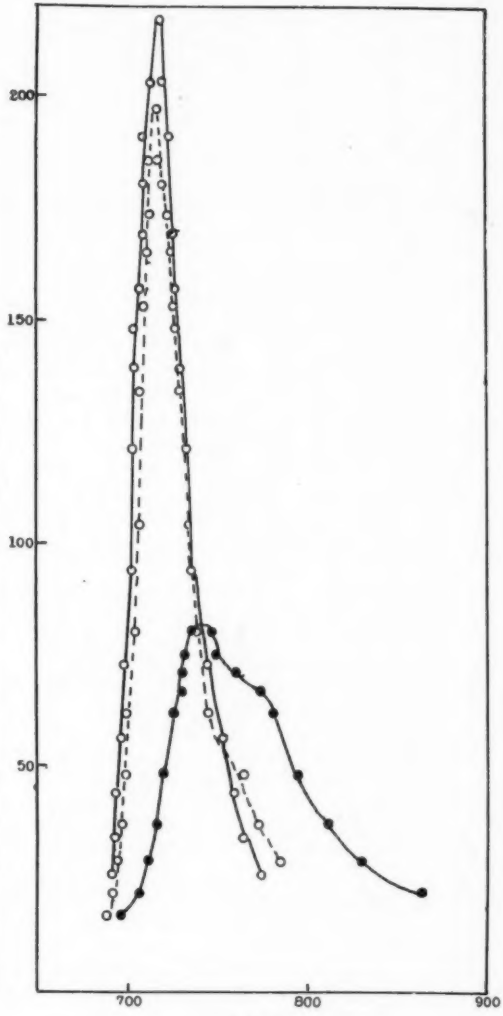


Fig. 2.

—o—o— Zinc-protoporphyrin in pyridine-chloroform.
—•—•— Zinc-protoporphyrin in water.
– – o – – o – – Zinc-protoporphyrin-globin.

Ordinates represent $\epsilon 10^{-3}$
Abscissae represent $\nu 10^{-12}$

$$\epsilon = \frac{M}{cd} \log \frac{I_0}{I}.$$

where M = Molecular weight of the pigment. c = No. of gm. of pigment in one litre. d = Depth of cell in cm. I_0 = Intensity of entering beam. I = Intensity of emergent beam.

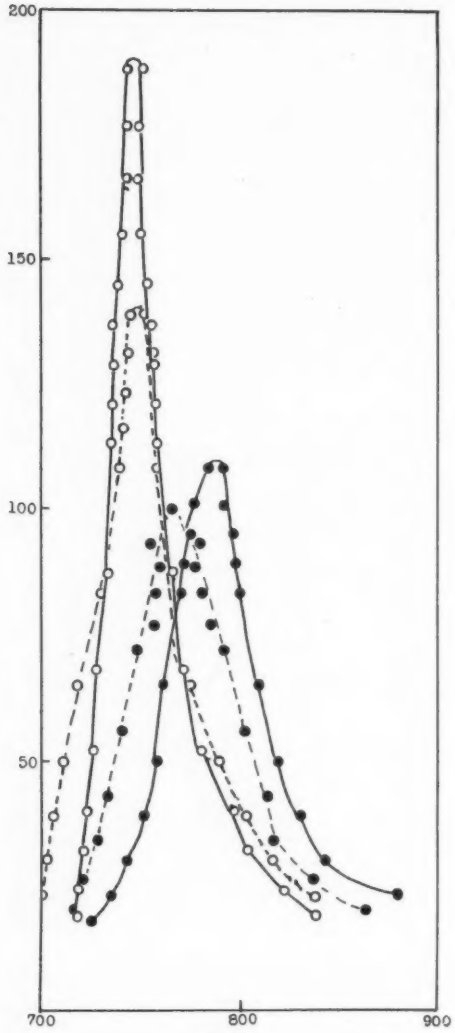


Fig. 3.

—o—o— Copper-protoporphyrin in pyridine-chloroform.
—●—●— Copper-protoporphyrin in water.
– – –o– – –o– – – Copper-protoporphyrin-globin.
– – –●– – –●– – – Copper-protoporphyrin with denatured globin.

Ordinates represent $\epsilon 10^{-3}$
Abscissae represent $\nu 10^{-12}$

$\epsilon = \frac{M}{cd} \log \frac{I_0}{I}$. (see legend to Fig. 1.)

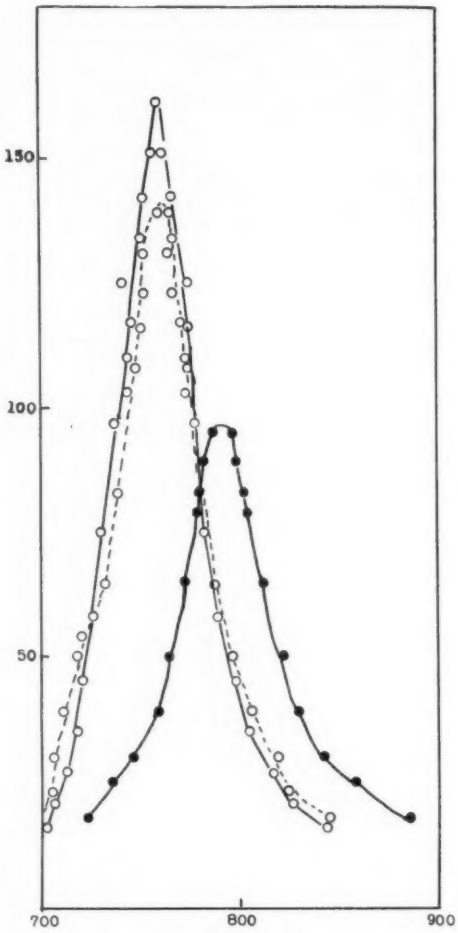


Fig. 4.

—o—o— Nickel-protoporphyrin in alcoholic hydrochloric acid.
—●—●— Nickel-protoporphyrin in water.
– – –o– – –o– – – Nickel-protoporphyrin-globin.

Ordinates represent $\epsilon 10^{-3}$
Abscissae represent $\nu 10^{-12}$

$\epsilon = \frac{M}{cd} \log \frac{I_0}{I}$. (see legend to Fig. 1.)

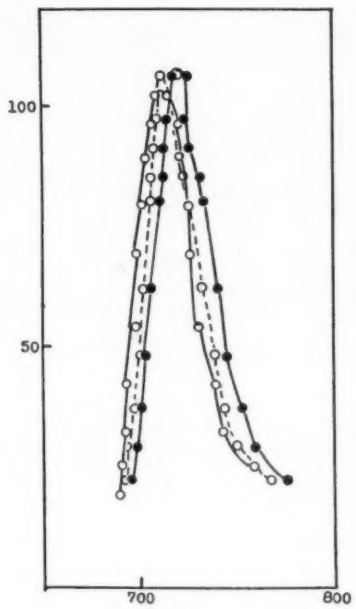


Fig. 5.

—o—o— Cobalt-protoporphyrin in pyridine-chloroform.

—•—•— Cobalt-protoporphyrin in water.

– – –o– – –o– – – Cobalt-protoporphyrin-globin.

Ordinates represent $\epsilon 10^{-3}$
Abscissae represent $\nu 10^{-12}$

$\epsilon = \frac{M}{cd} \log \frac{I_0}{I}$. (see legend to Fig. 1.)

The relative amounts of globin with which equivalent amounts of haematin, of copper-protoporphyrin and of protoporphyrin combine were determined by adding known amounts of the pigments to varying amounts of globin solution containing a little ammonia. The intensity of the absorption at suitable wave-lengths was read by means of a König-Marten spectrophotometer. The results indicated that while the first two pigments required about equal amounts of globin the protoporphyrin required only about half as much.

DISCUSSION.

The results set out in Table 1 show that haematin dissolved in organic solvents has a tendency to aggregate in particles having an ultra-violet absorption spectrum with the low diffuse band of haematin dissolved in water, whether in alkaline solution or with the help of a protective colloid as e.g. "acid haematin". This aggregating process appears to be promoted by the presence of inorganic ions, and the highest absorption coefficients were obtained in the presence of about 0·001 N. hydrochloric acid, when the haemin was first converted to haematin by sodium hydroxide. The height of the band however diminished very considerably in the course of two hours. At the same time the frequency of the head of the band approached that of acid haematin in water. In the presence of higher concentrations of acid the fall was more rapid. The temperature of the laboratory during these experiments was about 25° C. When the haemin was first dissolved in a mixture of pyridine and chloroform the initial height of the band was less than when sodium hydroxide was used, but the fall was less pronounced. A similar decrease was observed in the cases of copper and nickel. In the case of cobalt protoporphyrin, whose molecules showed in general less tendency to aggregate, a small decrease was seen when it had been dissolved in ammonia but none when it had been dissolved in the pyridine-chloroform mixture. It must not be forgotten that, from necessity, the other metallo-porphyrins were dissolved under those conditions under which haematin showed the slowest decrease in the height of the band. The relatively high band of protoporphyrin when in solution in alcoholic hydrochloric acid and the absence of any change in the band on standing suggest that in this solvent the porphyrin exists chiefly in the molecular state.

A comparison of the frequencies of the main bands of the ultra-violet spectra show definite divergences. In aqueous solution the bands of cobalt and of zinc protoporphyrins lie near to the band of oxyhaemoglobin while those of the other derivatives lie near that of haematin. On standing in aqueous solution the band of copper protoporphyrin diminished as did that of nickel, while those of cobalt and zinc protoporphyrins showed no measurable diminution during a similar period.

The bands of the globin compounds fall into two groups separated by over 150Å. The one group includes those of cobalt, zinc and ferrous protoporphyrins; the other those of protoporphyrin and its ferric, copper and nickel complexes.

TABLE 2.

The chief ultra-violet absorption band. Wavelengths and absorption coefficients.

Solvent.	Proto-porphyrin λÅ	Proto-porphyrin $\epsilon 10^{-3}$	Cu-proto-porphyrin λÅ	Cu-proto-porphyrin $\epsilon 10^{-3}$	Ni-proto-porphyrin λÅ	Ni-proto-porphyrin $\epsilon 10^{-3}$	Co-proto-porphyrin λÅ	Co-proto-porphyrin $\epsilon 10^{-3}$	Zn-proto-porphyrin λÅ	Zn-proto-porphyrin $\epsilon 10^{-3}$	Fe″-proto-porphyrin λÅ	Fe″-proto-porphyrin $\epsilon 10^{-3}$	Fe‴-proto-porphyrin λÅ	Fe‴-proto-porphyrin $\epsilon 10^{-3}$
Water	3,750	62	3,825	108	3,785	99	4,160	112	4,040	83	3,935	44	3,850	49
Pyridine-chloroform	4,045	120	4,020	194	3,965	161	4,220	109	4,200	217	—	—	4,015	38
Alcoholic HCl	4,105	197	3,975	167	3,960	161	4,180	102	—	—	—	—	3,960	173
Globin solution	4,020	114	4,005	139	3,950	136	4,190	110	4,200	194	4,200	123	4,040	162
20 p.c. pyridine in water	4,030	104	—	—	—	—	—	—	—	—	—	—	—	—
Denatured globin solution	4,070	74	3,920	100	—	—	—	—	—	—	4,240	110	—	—

In aqueous solution only Co-pph and Zn-pph have any considerable proportion in the state of single molecules. The band of reduced haematin is low and broad like that of alkaline haematin, though of somewhat longer wavelength. The band of haematin in pyridine-chloroform shows a small proportion in the state of free molecules. The values for haematin, reduced haematin and for protoporphyrin-denatured globin are added for comparison from previous work.

The metallo-porphyrin globin compounds appeared to be quite stable for some hours even in solutions as alkaline as pH 9·5. Repeated photographs revealed no diminution in the height of the main ultra-violet band, and the height of the band when combination was effected at pH 9·5–10 was as great as when combination was effected at pH 8–8·5.

The α bands of the visible spectra of these metallo-protoporphyrins differ from those of the corresponding globin compounds by an amount which depends on the metal present and which falls more or less in line with the other indications of the degree of association of the free metallo-porphyrin. Except in the case of zinc the α bands of the globin compounds have a shorter wave-length than those of the corresponding free metallo-protoporphyrins. That of zinc is 20Å longer. The difference between zinc protoporphyrin and its globin compound is, however, unmistakable from the relative sharpness of the bands of the latter, its enhanced transmission in the green region and the difference in the ultra-violet absorption spectra.

TABLE 3.

The bands of the visible spectra. Wavelengths in mμ.

Solvent.	Protoporphyrin.				Cu-proto-porphyrin.		Ni-proto-porphyrin.		Co-proto-porphyrin.		Zn-proto-porphyrin.	
	α	β	γ	δ	α	β	α	β	α	β	α	β
Water	636				578	538	567	527	573	536	586	547
Pyridine-chloroform	631	576	560	507	571	533	564	524	571	535	589	549
Alcoholic HCl	610	568			570	532	560	521	569	534	—	—
Globin solution	622	568	543	509	569	530	559	520	568	533	587	549

Except in the case of zinc, and of acid protoporphyrin the wavelength of the bands decreases in the order water-chloroform, alcoholic HCl, globin, suggesting parallel changes in the degree of association of the molecules or of interference with one another's absorption. The positions of the bands of alkaline protoporphyrin show great variations with varying concentrations of neutral salts and values still further to the red are frequently noted.

The absorption spectrum of protoporphyrin dissolved in alcoholic hydrochloric acid differs greatly from that of the porphyrin dissolved in water containing a little ammonia and pyridine. Even when dissolved in 20 p.c. pyridine in water its spectrum suggests that perhaps only half of its molecules are indepen-

dent, while in the solution containing little pyridine very few can be so. Notwithstanding this, when protoporphyrin so dissolved is added to globin as much protoporphyrin globin appears to be formed as when the protoporphyrin is dissolved in alcoholic hydrochloric acid. It is also certain that protoporphyrin readily assumes a form that does not react at all with globin but whose spectrum differs from that of the form in pyridine-ammonia chiefly in some decrease in the height of the ultra-violet band and an increase in the wave-lengths of the visible bands. The intermediate form seems to be in some way comparable with the state of alkaline haematin, in which a very small amount of independent molecules appears to be in dynamic equilibrium with a large portion which is aggregated but capable of dissociation when the equilibrium is in any way disturbed. It would appear, however, that only about half the protoporphyrin exists in this intermediate form, and this view is borne out by the spectrophotometric titrations of it and haematin against the same solution of globin.

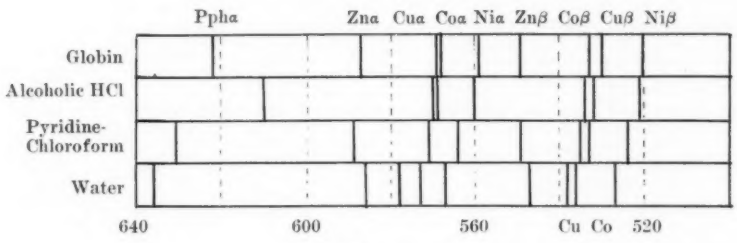


Fig. 6.

The visible spectra of protoporphyrin and some metallo-protoporphyrins. See Table 3.

The figures below the graph represent wavelengths in mμ. Except for the one instance noted below the graph the bands in all the solvents follow the order above the graph. Pph = protoporphyrin. The elementary symbols refer to the metallo-protoporphyrin yielding the bands. α and β indicate the α and β bands of the visible spectrum.

This class of work still suffers much from lack of a suitable solvent for the pigments used which accounts, in part, for the paucity of experiments in this field and renders the interpretation of many results very difficult. Pyridine, except in minute concentration, is so deleterious to native proteins and to globin in particular that its use seemed excluded. The same is true of alcohol. The combined effects of low concentrations of pyridine and ammonia in water in dissolving metallo-protoporphyrins rendered these experiments possible. In general about 0·25 c.c. of 12 p.c. aqueous pyridine and 0·15 c.c. of normal ammonia readily dissolved a milligramme of any of the pigments studied, which would have required several times as concentrated a pyridine solution alone and which were totally insoluble in aqueous alkalies. It is interesting to notice indications of compounds analogous to haemochromogens on the part of manganese mesoporphyrin (Clark, 1938), and it is difficult to avoid the idea of some such compounds existing among the metallo-protoporphyrins. If so they appear to differ sharply from ferro-haemochromogens in solubility. The diminution in the height of the absorption band of protoporphyrin globin in alkaline solution has again been observed when the porphyrin was added in alcoholic solution but not when added in pyridine-ammonia water. It would seem probable that it must be attributed to the known enhanced denaturant effect of alcohol in alkaline solution. None of the metallo-porphyrins showed a similar effect.

Whatever be the part played by the iron atom, apart from its power of uniting with gases, in linking the porphyrin molecule to globin it would appear possible to form compounds essentially similar to haemoglobin with other metals in place of iron, or indeed without any metal at all.

SUMMARY.

The ultra-violet absorption bands of the protoporphyrin compounds of copper, nickel, cobalt and zinc are measured in organic solvents, in water, and in the presence of globin. The significance of the differences in the spectra is discussed.

Acknowledgments. The ultra-violet spectrophotometer is the property of the Australian National Health and Medical Research Council. The König-Marten spectrophotometer was kindly lent by Professor Wright, of the University of Melbourne.

REFERENCES.

Anson, M. L. and Mirsky, A. E. (1930): J. gen. Physiol., 13, p. 469.
Clark, W. M. *et alii* (1938): C.R. Trav. lab. Carlsberg, sér. chim., 22, p. 129.
Fischer, H. and Putzer, B. (1926): Z. physiol. chem., 154, p. 39.
Hill, R. and Holden, H. F. (1926): Biochem. J., 20, p. 1326.
Holden, H. F. (1936): Austral. J. exp. Biol., 14. p. 291.
Holden, H. F. (1937): *Ibid.*, 15, p. 409.

APPENDIX.

DETAILS OF SOLUTIONS FOR PHOTOGRAPHY.

Throughout this section the following abbreviations are used:

Pph = protoporphyrin.
Alc. HCl = 1 c.c. concentrated aqueous hydrochloric acid diluted to 100 c.c. with absolute alcohol.
NH_3 = 1 c.c. ·880 ammonia diluted to 20 c.c. with water.
Globin = a solution of ox globin of approximately 0·5 p.c.
Py = pyridine.

Fig. 1. 2·21 mg. pph in 100 c.c. alc. HCl.
3·47 mg. pph, 0·8 c.c., 12 p.c. py in H_2O, 0·4 c.c. NH_3, H_2O to 10·0 = stock.
0·8 c.c. stock, H_2O to 10·0 c.c., pH 9·4.
0·8 c.c. stock, 2 c.c. globin H_2O to 10·0 c.c., pH 8·9.

Fig. 2. 0·93 mg. Zn-pph dissolved in 0·5 c.c. 20 p.c. py in $CHCl_3$, add $CHCl_3$ to 10·0 c.c. = stock.
0·9 c.c. stock, $CHCl_3$ to 5·0 c.c.
1·27 mg. Zn-pph, 0·3 c.c. 12 p.c. py in H_2O, 0·2 c.c. NH_3, H_2O to 10·0 c.c. = stock.
2·0 c.c. stock, H_2O to 10·0 c.c., pH 9·8.
2·0 c.c. stock 2·0 c.c. globin, H_2O to 10 c.c., pH 8·3.

Fig. 3. 1·46 mg. Cu-pph, 0·2 c.c. py, $CHCl_3$ to 10·0 c.c. = stock.
0·8 c.c. stock, $CHCl_3$ to 5·0 c.c.
1·22 mg. Cu-pph, 0·3 c.c. 12 p.c. py in H_2O, 0·2 c.c. NH_3, H_2O to 10·0 c.c. = stock.
2·0 c.c. stock, H_2O to 10·0 c.c., pH 9·6.
2·0 c.c. stock, 2·0 c.c. globin, H_2O to 10·0 c.c., pH 8·1.
1·09 mg. Cu-pph, 0·3 c.c. 12 p.c. py in H_2O, 0·2 c.c. NH_3, H_2O to 10·0 c.c. = stock.
the denatured globin solution contained 4·6 mg. denatured rabbit globin per c.c. in 0·01 N HCl.
1·0 c.c. denatured globin solution, 0·5 c.c. NH_3, 1·0 c.c. stock, H_2O to 5·0 c.c., pH 10·6.

Fig. 4. 1·35 mg. Ni-pph, 0·2 c.c. py, $CHCl_3$ to 10·0 c.c. = stock.
1·0 c.c. stock, alc. HCl to 5·0 c.c.
1·51 mg. Ni-pph, 0·4 c.c. 12 p.c. py in H_2O, 0·2 c.c. NH_3, H_2O to 10·0 c.c. = stock.
1·6 c.c. stock, H_2O to 10·0 c.c.
1·6 c.c. stock, 1·0 c.c. NH_3, 2 c.c. globin, H_2O to 10·0 c.c.

Fig. 5. 1·41 mg. Co-pph, 0·2 c.c. py, $CHCl_3$ to 10·0 c.c. = stock.
0·8 c.c. stock, $CHCl_3$ to 5·0 c.c.
0·98 mg. Co-pph, 0·4 c.c. NH_3, H_2O to 10·0 c.c. = stock.
1 c.c. stock, H_2O to 5·0 c.c.
1 c.c. stock, 1 c.c. globin, H_2O to 5·0 c.c.

OBSERVATIONS ON STAPHYLOCOCCAL HAEMOLYSINS AND STAPHYLOCOCCAL LIPASE.

by R. CHRISTIE AND J. J. GRAYDON

(From the Commonwealth Serum Laboratories, Melbourne.)

(Accepted for publication 25th November, 1940.)

The α and β toxins or haemolysins of staphylococci have recently received much attention. They occur in cell-free filtrates, and are antigenically distinct (Glenny and Stevens, 1935; Bryce and Rountree, 1936). The α toxin lyses rabbit and sheep red blood cells at 37° C.; Morgan and Graydon (1936) demonstrated two components, $\alpha 1$ and $\alpha 2$, recognizable by their different combining powers with antitoxins containing different proportions of the respective antibodies. The β toxin does not lyse rabbit cells and only lyses sheep cells when incubation at 37° C. is followed by refrigeration. This action of the β toxin on sheep cells is called "hot-cold" lysis.

Bryce and Rountree used "spot-inoculation" of sheep blood agar plates to identify the toxins of staphylococci. After 18–24 hours' incubation at 37° C., colonies of α toxin-producers were surrounded by a zone of complete clearing; β toxin-producers were surrounded by a zone of darkening which became lighter on refrigeration. Strains producing both toxins showed a combination of these two effects, an inner zone of complete clearing surrounded by a darker zone which became lighter on cooling.

Orcutt and Howe (1922) found evidence of lipase-production by staphylococci on blood agar plates containing cream. Colonies of certain staphylococci were surrounded by a zone of clearing although no haemolysis was visible when the organisms were grown on blood agar plates without cream. They showed that staphylococci may form an extra-cellular thermolabile enzyme which hydrolyses butter-fat, and that the fatty acid (or soap) formed produces the haemolysis on cream blood agar shown by these strains.

Birch-Hirschfeld (1937), using hydrolysis of tributyrin to estimate lipase and sheep cell lysis to estimate haemolysin, examined the relationship between lipase and haemolysin production by 13 strains of staphylococci. The ratio between the amounts of lipase and haemolysin was fairly constant, from which it was deduced that they had a common origin.

Observations made in this laboratory on the lipolytic and haemolytic power of staphylococci are interesting in view of possible interpretations they offer concerning the action of α toxin, β toxin and lipase on sheep red cells.

In the following report the phrase "zone of β haemolysis", unless otherwise indicated by the context, refers to the zone of darkening produced on sheep blood agar by β toxin, although, strictly speaking, this is not a zone of lysis.

Experiments and Results.

(a) *Detection of lipase production by staphylococci.* Blood agar plates, containing 0·2 p.c. cream, were inoculated with various strains of staphylococci and incubated at 37° C. Control plates without cream were similarly treated. Lipase-production, as indicated by increased haemolysis on the plates containing cream was quite distinct with some strains, less so with others. Incubation for more than 24 hours was usually necessary before any increase became apparent. Horse and rabbit blood indicated lipase-production earlier than sheep or human blood.

Uncertain results were obtained with strongly haemolytic strains producing only a small quantity of lipase.

More direct and less equivocal results were obtained with nutrient agar plates containing 0·1 p.c. washed butter-fat and 0·005 p.c. neutral red. The butter-fat was washed six times by shaking with hot water and centrifuging. On this medium fat hydrolysis was indicated by a zone round each colony (Fig. 1), in which the fat particles were red, whereas those in the rest of the medium were yellow. Twenty-four pathogenic and twenty-four non-pathogenic strains (using ability to coagulate plasma as the criterion of pathogenicity) were cultivated on these plates and examined daily for nine days. With most lipolytic strains red zones appeared within two days, with others not until the fourth day; no appreciable increase occurred after the fifth day. The haemolytic power of the strains was determined from the appearance of their colonies on sheep-blood agar after 24 hours' incubation at 37° C. and 18 hours' refrigera-

tion at 4° C. The results of these tests are shown in Table 1. Lipase is produced independently of haemolysin and appears to have no connection with pathogenicity.

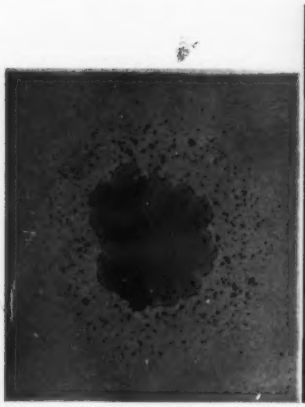
TABLE 1.

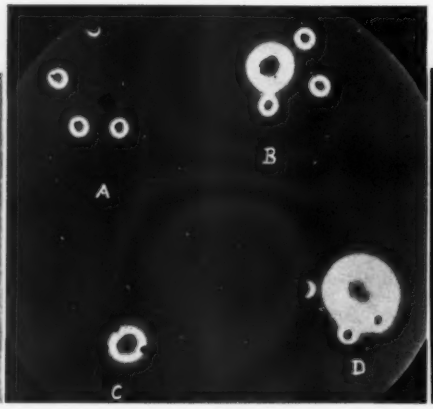
Results of haemolysin and lipase tests, with number of strains in each group.

	Pathogenic strains.				Non-pathogenic strains.	Total.
Type of haemolysis	α	αβ	β	Nil	Nil	
Lipase positive	7	12	–	1	17	37
Lipase negative	1	1	1	1	7	11
Total	8	13	1	2	24	48

Lipolytic activity was much more marked with strain S36 than with any other. Since this strain played a large part in the experiments which followed, its other characteristics were examined.

(b) *Particulars of strain S36.* This organism, though pigmented, was apparently a non-pathogen. It produced little or no haemolysis on sheep-blood agar after 24 hours' incubation at 37° C. No α or β toxin was detected when it was grown under increased CO_2 tension in broth containing 0·1 p.c. agar. It did not ferment mannitol, was not agglutinated by antisera prepared from serological types of pathogenic strains and did not coagulate plasma.





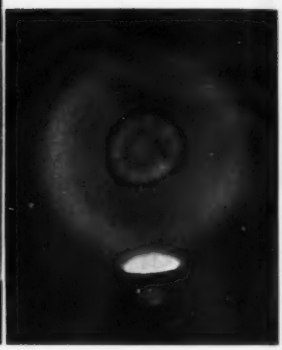


Fig. 1. **Fig. 2.** **Fig. 3.**

Fig. 1. Colony of a lipolytic staphylococcus (strain S36) on butter-fat neutral red agar after 48 hours' incubation at 37° C. The dark particles (actually red) consist of butter-fat partly hydrolysed by lipase.

Fig. 2. (a) A colony of staphylococci (strain S32a) on sheep blood agar surrounded by two concentric zones of β haemolysis. Four colonies of a non-haemolytic lipolytic staphylococcus (strain S36) are causing clearing in these zones. Three colonies of strain S36 producing no haemolysis appear in the toxin-free parts of the plate.

(b) Colony of staphylococci on sheep blood agar showing a clear zone of α haemolysis surrounded by two zones of β haemolysis. Three colonies of strain S36 appear in the zones of β haemolysis.

(c) A colony of α toxin-producing staphylococci (strain S210) on sheep blood agar showing three colonies of strain S36 within the area affected by α toxin.

(d) Colony of staphylococci on sheep blood agar showing a large zone of α haemolysis surrounded by a zone of β haemolysis. Three colonies of strain S36 appear within the zones of haemolysis.

Fig. 3. Colony of staphylococci (strain S32a) on sheep blood agar, surrounded by a large zone of β haemolysis showing the clearing caused within this zone by the lipase from the colony of lipolytic staphylococci (strain S36).

Injected intravenously and intraperitoneally into rabbits and mice in large doses it produced no ill effect. It showed some relationship to the pathogenic staphylococci in its susceptibility to lysis by the staphylococcal bacteriophages, Au I, Au II, Au III and Au IV (Burnet and Lush, 1935).

Its lytic action on red cells in cream blood agar was not due to fermentation of lactose or casein present, since it did not ferment lactose, gave no zone of clearing on fat-free milk agar such as proteolytic strains produce, and did not liquefy gelatine (28 days allowed).

(e) *The effect of staphylococcal lipase on sheep red cells previously altered by β toxin.* One strain (S32a, received from Dr. L. M. Bryce) producing β toxin but no α toxin showed no signs of lipolytic activity when tested by the method of Orcutt and Howe or when grown on butter-fat agar. A mixed suspension of this strain and the lipolytic strain S36 was plated out on sheep-blood agar. After 24 hours' incubation typical zones of β haemolysis formed round the colonies of S32a. No haemolysis showed round the colonies of S36 in the parts of the medium free of β toxin, but when within zones of β haemolysis they were surrounded by an area of clearing.

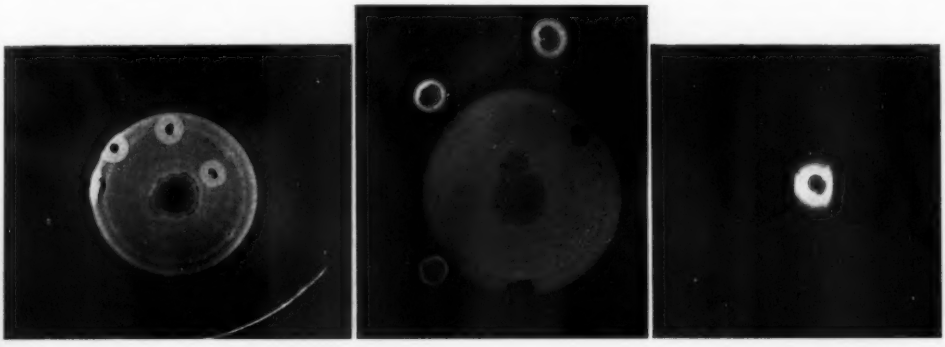


Fig. 4. **Fig. 5.** **Fig. 6.**

Fig. 4. Colony of staphylococci (strain S32a) on sheep blood agar surrounded by zones of β haemolysis, showing the clearing caused within these zones by a non-haemolytic air-borne cocco-bacillus. Five colonies of the latter appear.

Fig. 5. Colony of staphylococci (strain S32a) on sheep blood agar surrounded by two zones of β haemolysis. Five colonies of a strain (S210) producing α toxin show zones of clearing only when they are beyond the area of β haemolysis.

Fig. 6. Colony of α toxin-producing staphylococci (strain S210) on sheep blood agar with two colonies of β toxin-producing staphylococci (strain S32a) showing the extra clearing produced where β toxin acts on cells already affected by α toxin.

Fig. 2(a) illustrates such an effect. The growth in the centre is from strain S32a. It is surrounded by two concentric zones of β haemolysis. The inner one was formed within 24 hours, and was partly lightened by the cooling which occurred when the plate was withdrawn from the incubator for examination and spot-inoculation with strain S36. Three colonies of S36 showing clear zones round them can be seen within the area affected by β haemolysin. A fourth colony on the outer edge of the β zone shows clearing only on the segment falling within that zone, with no change in the medium on the other side of the colony. Three colonies of S36, which can be seen on toxin-free parts of the medium in Fig. 2 show no haemolysis. Clearing round colonies of S36 also occurred within the zones of β haemolysis surrounding colonies of αβ strains (Fig. 2, b and d).

Fig. 3 shows the effect produced when an otherwise invisible zone of lipase overlapped a zone of β haemolysis. The lipase lysed the cells affected by β toxin but did not lyse normal cells. This plate also shows the lightening in colour of the cells within the zone of β haemolysis which occurred on partial cooling.

Since the combined action on sheep red cells of β toxin and the exudate from the lipolytic non-toxigenic strain gave a result resembling the action of α toxin, the possibility that this exudate converted β toxin into α toxin was examined. Strains S32a and S36 were grown

separately and together, for four days, under increased CO_2 tension in broth containing 0·1 p.c. agar. The cultures, after centrifugation, were titrated against rabbit and sheep red cells. The absence of α toxin was indicated by the absence of rabbit-cell lysin in all the filtrates. The presence of β toxin, indicated by sheep cell lysis after one hour's incubation followed by refrigeration, was demonstrated in the mixed culture and in the pure culture of strain S32a. The centrifugates of the two pure cultures were then mixed, filtered through a collodion membrane (pore size 380mμ) and incubated overnight at 37° C. Titration against rabbit and sheep cells showed the presence of β toxin only.

A skin staphylococcus, strain S205, although non-lipolytic, produced clearing in zones of β haemolysis. This suggested that the exudate responsible for the clearing produced by strain S36 was not necessarily lipase. However, comparison of strains S205 and S36 indicated that the clearing caused by the former was due to a different action. The following points of difference were noted: (1) Strain S205 liquefied gelatin whereas S36 did not. (2) The zones of clearing produced by S36 in zones of β haemolysis were faintly opaque and had an indefinite boundary, whilst those produced by S205 were glassy clear and had a sharp boundary. Many air-borne chromogenic bacilli also produce glassy clear, sharply defined zones of clearing in zones of β haemolysis (Fig. 4); two such strains were examined and found to be proteolytic but not lipolytic. (3) Strain S205 and these two air-borne bacilli caused clearing in zones of partial haemolysis round colonies of Cl. welchii on sheep blood agar, whereas strain S36 caused no clearing whatever. Results obtained with lipolytic and non-lipolytic α toxin-producing strains (Section (e)) agreed with the hypothesis that lipase caused clearing in zones of β haemolysis. The conclusion, therefore, was that the clearing produced by S36 was lipolytic in nature, and that produced by S205 was probably due to proteolysis.

(d) *Effect of staphylococcal lipase on cells already attacked by α toxin.* An α toxin-producing strain, S210, non-lipolytic when tested on cream blood agar or butter-fat agar, was spot-inoculated on sheep blood agar. After 24 hours' incubation at 37° C. the growth was surrounded by a zone of α lysis. This zone was almost colourless but had a slight opacity and an indefinite boundary. Microscopic examination showed that there were no red cells immediately surrounding the colony, and that the number of cells remaining within the zone of α lysis increased with distance from the colony until unaltered medium was reached.

The lipolytic strain, S36, was inoculated on and near this zone of clearing and a further 24 hours' incubation at 37° C. allowed. No increase of clearing was observed (Fig. 2c). This indicated that the modification in the cell induced by β toxin was not merely an incomplete stage of that induced by α toxin.

TABLE 2.

Results of haemolysin titrations showing the inhibitory effect of β toxin on the lytic action of α toxin on sheep red cells.

Series I. Eight drops of each diluted preparation of β toxin were added to the corresponding tube. One drop of 10 p.c. sheep red cell suspension was added to each tube. After one hour's incubation at 37° C., one drop of saline was added to each tube.

Haemolysis after one hour's further incubation at 37° C. is shown in row H (= hot).

Haemolysis after overnight refrigeration is shown in row C (= cold).

Series II. Similar to the above except that one drop of α toxin was added in place of one drop of saline.

Series III. Similar to Series II except that the drop of α toxin was added to each tube immediately prior to the addition of the drop of sheep cell suspension. Readings were made after one hour at 37° C. and after overnight refrigeration.

Final dilution of β toxin.

			1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Series	I	H	—	—	—	—	—	—	—	—	—	—
		C	++++	++++	++++	++++	++++	++++	+++	++	±	—
Series	II	H	—	—	±	±	±	—	—	++	++++	++++
		C	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Series	III	H	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
		C	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

++++ indicates complete haemolysis.
+++ „ 75% „
++ „ 50% „
± „ trace of „ (less than 5%).

(e) *Inhibition of α haemolysis by β toxin.* A zone of β haemolysis was produced on sheep blood agar by inoculating with strain S32a and incubating for 24 hours. The non-lipolytic α toxin-producing strain, S210, was inoculated on and near this zone. After 24 hours' further incubation the colonies of S210 were surrounded by typical zones of α haemolysis only when they were beyond the zone of β haemolysis; when within the zone of β haemolysis there was no sign of clearing round them (Fig. 5). Even after refrigeration overnight no indications of lytic action due to α toxin occurred round the colonies of S210 within the partly lysed zone of β haemolysis.

A lipolytic strain, S61, which produced α toxin but no β toxin, was treated in the same way as strain S210. Its colonies were surrounded by large typical zones of α lysis when they occurred in medium free of β toxin; colonies situated within β areas had much smaller zones due to the action of the lipase produced by strain S61.

Inhibition of α haemolysis by β toxin was also demonstrated in test-tube experiments using cell-free filtrates. Eight drops of successive doubling dilutions of β toxin were added to a row of 3½ in. x ½ in. test-tubes. One drop of 10 p.c. washed sheep cell suspension was added to each tube and the rack placed in a water-bath at 37° C. After an hour one drop of saline was added to each tube. Readings of haemolysis were made after incubation for another hour and again after over-night refrigeration. A second row was prepared like the first, but with one drop of a diluted α toxin substituted for the drop of saline. (The amount of α toxin added was shown by preliminary titration to be double that required to lyse the cells completely.) A third series of tests was performed in which the α toxin was added to the β toxin preparations just prior to the addition of the cell suspension.

The results are shown in Table 2. Haemolysis occurring after one hour's incubation at 37° C. was due to the action of α toxin. Further haemolysis on cooling indicated the action of β toxin. The results in Series I showed the degree to which the β toxin could be diluted before it ceased to cause "hot-cold" lysis. The results in Series II showed that the α toxin could only lyse the cells previously treated with β toxin when the concentration of the latter was too low to affect them. Series III showed that the mere presence of β toxin did not inhibit the lytic action of α toxin.

(f) *Effect of β toxin on cells already attacked by α toxin.* The α toxin-producing strain, S210, was inoculated on sheep blood agar. After 24 hours' incubation at 37° C. a zone of clearing with the slight opacity and indefinite boundary described in Section (d) appeared around the colony. The β toxin-producing strain S32a was inoculated on the medium near this zone. After 24 hours' further incubation at 37° C. a zone of β haemolysis had formed round the growth from strain S32a. This zone overlapped the zone of α lysis. The overlapped area was distinct from the remainder of the zone round each growth in that it was glassy-clear. The arc of the circumference of the zone of β haemolysis in this area was sharply defined by the contrast between the opacity present in the zone of α lysis on the outside and the glassy clearness within (Fig. 6). On microscopical examination, the area which appeared glassy-clear to the naked eye was slightly opaque, but there were no unlysed cells present as in the normal zone of α lysis. That β toxin could lyse such cells showed that some change had been induced in them by α toxin since β toxin will not lyse normal sheep red cells at 37° C.

It was also noticed that the zones of α lysis occurring on sheep blood agar round colonies of αβ strains differed from those round colonies of α strains producing no β toxin; they were glassy-clear, had a sharp boundary. Such zones therefore should not be regarded as zones of α lysis but of (α + β) lysis.

The action of β toxin in augmenting the action of α toxin on sheep cells was also shown using cell-free filtrates. A drop of α toxin produced a slightly opaque zone of clearing on a sheep blood-agar plate after one hour at 37° C. A drop of β toxin was allowed to fall on the edge of this zone. After one hour's further incubation the area of α lysis overlapped by the drop of β toxin was much clearer than the area of α lysis alone.

(g) *The action of the toxins on the colouring matter of the red cells.* Several small experiments showed that the loss of colour in zones of α lysis round colonies on blood agar was partly or completely inhibited when diffusion of haemoglobin from these zones was prevented. No visible change occurred around colonies grown on lysed blood agar plates. A blood agar plate flooded with α toxin showed complete lysis but no loss of colour. Rigdon (1939) reported that staphylococcal toxin had no effect on lysed blood agar, although it caused areas of clearing on normal blood agar.

Spectroscopic examination of a sheep red cell suspension lysed by α toxin did not show absorption bands of altered haemoglobin. A red cell suspension treated at 37° C. for one hour with β toxin, and also a saline extract from sheep blood agar which had been discoloured by treatment for one hour with β toxin, showed no indication of the presence of altered haemoglobin. However, a red cell suspension left in contact with β toxin at 37° C. overnight and then lysed by cooling showed the absorption bands of methaemoglobin. It seems probable that this methaemoglobin resulted from some secondary reaction not necessarily involving

the toxin. When a zone of β haemolysis on sheep blood agar is viewed obliquely by reflected light it appears lighter than the rest of the medium, suggesting that the darkening seen with transmitted light is due to a change in the opacity of the cells rather than to an alteration in the colouring matter.

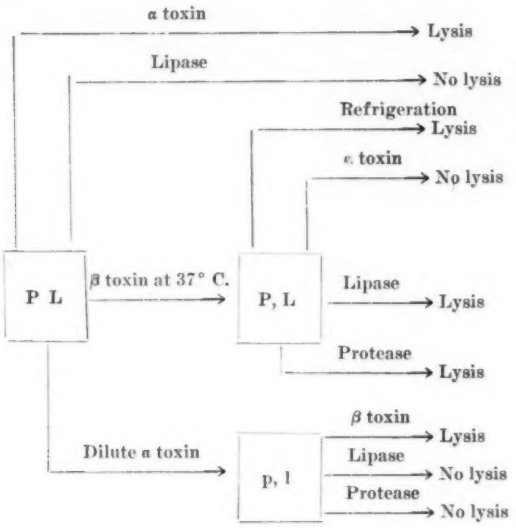
DISCUSSION.

As Rigdon has reported, staphylococcal α toxin does not attack the haemoglobin of the red cell. The loss of colour which occurs round colonies of staphylococci on blood agar is due to diffusion of the liberated haemoglobin into the surrounding medium in a manner similar to that described by Brown (1919) to explain the clearing round colonies of streptococci. The destruction of haemoglobin found by Clauberg (1929) in test-tube experiments, by which he explained the clearing on blood agar, was possibly due to further bacterial action.

From general considerations it would appear that α and β toxins are somewhat similar substances, capable of linking up with some substrate in the cell envelope. This occurs primarily as an adsorption (Levine, 1938; Forssman, 1939), following which the mutual relationship between the protein and lipoid of the cell envelope is modified according to the nature of the toxin. The β toxin alters the protein-lipoidal material so that it is susceptible to staphylococcal lipase and to some proteolytic enzymes having no apparent effect on the normal cell. That cells treated with β toxin are no longer lysed by α toxin may be due to this alteration in the substrate normally attacked by α toxin. It could also be explained by assuming that the β toxin has occupied most of the available linkages in the substrate, leaving insufficient points of attack for the α toxin. Such an explanation has a parallel in the case of inhibited adsorption catalysis and in the "blockade immunity" hypothesis evolved to explain the action of weakly lytic bacteriophages in protecting against related and more potent bacteriophages (White, 1937). A further possibility is that, after attack by β toxin, the permeability of the envelope is so altered that α toxin is prevented from reaching the internal structure of the envelope, which we must then presume to be its point of attack. The change in the opacity of the cell wall and the subsequent formation of methaemoglobin, though not inconsistent with either of the other theories, support the theory of altered permeability. From experiments with toxic filtrates on sheep blood agar, it appears that the action of β toxin on the envelopes of the red cells is completed rapidly. This conclusion is supported by the almost instantaneous lysis which follows rapid cooling of a suspension of sheep cells treated for one hour with β toxin. This lysis seems to be the result of a secondary change, possibly purely physical, and not caused by further action of the toxin.

The microscopic examination of red cell suspensions and of blood agar plates treated with α toxin shows that there is a variation in the resistance of individual cells to haemolysis. The resistant cells are altered, however, by the α toxin so that they are haemolysed by β toxin. This increase in the haemolytic power of β toxin suggests the presence of an activator, possibly the lecithin of the cell, which exerts a markedly accelerating effect on haemolysis of red cells, as demonstrated with snake venoms and certain other haemolytic agents (Herrmann and Rohner, 1925; Kellaway and Williams, 1933).

Our results showing the effect of these staphylococcal exudates on sheep red cells may be summarized diagrammatically as under:



P L	represents the normal sheep red cell.
P, L	represents the sheep red cell with protein-lipoid relationship modified by β toxin.
p, l	represents the sheep red cell with protein-lipoid modified by α toxin.

Our inability to confirm the correlation found by Birch-Hirschfeld between lipase and haemolysin production may be due to the larger number of strains in our series or to differences in technique.

SUMMARY.

A simple method of detecting lipase production by staphylococci is described.

In an examination of 48 strains of staphylococci, lipase was produced independently of haemolysin. There was no correlation between lipase production and pathogenicity.

Using blood agar plates it was shown that staphylococcal lipase will lyse sheep red cells treated with staphylococcal β toxin, although it will not lyse normal sheep red cells; it does not augment the lytic action of staphylococcal α toxin.

Using blood agar plates and also by test-tube experiments with toxic filtrates it was shown that sheep red cells, after treatment with staphylococcal β toxin, are resistant to the lytic action of staphylococcal α toxin.

On the other hand it was shown that under suitable conditions staphylococcal β toxin, which does not lyse normal sheep cells, will lyse sheep cells previously treated with α toxin.

The mode of action of staphylococcal toxins on sheep red cells is discussed.

Acknowledgments. We are grateful to Dr. F. G. Morgan, Director of these Laboratories, and to Dr. F. M. Burnet, of the Walter and Eliza Hall Institute, for helpful advice and criticism, and also to Mr. F. J. Dempster for preparing the photographs.

REFERENCES.

Birch-Hirschfeld, L. (1937): Z. Hyg. Infekt Kr., 119, p. 431.
Brown, J. H. (1919): Monograph, Rockefeller Inst. for Med. Res., No. 9.
Bryce, L. M. and Rountree, P. M. (1936): J. Path. Bact., 43, p. 173.
Burnet, F. M. and Lush, D. (1935): J. Path. Bact., 40, p. 455.
Clauberg, K. W. (1929): Z. Hyg. Infekt Kr., 110, p. 442.
Forssman, J. (1939): Acta Path. et Microbiol. Scand., 16, p. 335.
Glenny, A. T. and Stevens, M. F. (1935): J. Path. Bact., 40, p. 201.
Herrmann, F. and Rohner, M. (1925): Arch. exp. Path. Pharmak., 107, p. 192.
Kellaway, C. H. and Williams, F. E. (1933): Austral. J. exp. Biol., 11, p. 81.
Levine, B. S. (1938): J. Immunol., 35, p. 131.
Morgan, F. G. and Graydon, J. J. (1936): J. Path. Bact., 43, p. 385.
Orcutt, M. L. and Howe, P. E. (1922): J. exp. Med., 35, p. 409.
Rigdon, R. H. (1939): J. lab. clin. Med., 24, p. 1264.
White, P. B. (1937): J. Path. Bact., 44, p. 276.

TISSUE INJURY BY THE TOXIN OF *CL. WELCHII*, TYPE A

by C. H. KELLAWAY AND E. R. TRETHEWIE[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Accepted for publication 12th December, 1940.)

In infections by *Cl. welchii* type A much of the resulting damage to the tissues must be attributed to the action of the toxin, which injected intravenously into animals causes petechial haemorrhages in the heart and alimentary tract, and sometimes haemorrhagic oedema of the lungs. The toxin is haemolytic *in vivo* and *in vitro*, and injected locally causes necrosis in skin and muscle, and oedema, often haemorrhagic, in the subcutaneous tissues. The toxin of *Cl. welchii* type D, which causes similar injuries to the tissues, liberates histamine and adenyl compounds from perfused organs (lung and liver) and adrenalin from the suprarenal glands of the intact animal (Kellaway, Trethewie and Turner (1940)). In general, poisons and toxins which cause cell injury also cause contraction of smooth muscle and the active substances liberated by injury contribute, in some cases at least, to the contraction. Buttle and Trevan (1928) and Nicholson (1934) have shown that type A toxin causes smooth muscle to contract.

In this paper we record observations on the effect of type A toxin in causing the liberation of pharmacologically active substances from perfused organs (lung, liver and hind limb) and of adrenalin from the suprarenal glands. We have also studied the action of the toxin on several kinds of smooth muscle.

METHODS.

For most experiments we used a toxin from the Commonwealth Serum Laboratories with a lethal dose of 0·07 mg. per 20 gm. mouse. Using defibrinated blood diluted with saline buffered to pH 7·5 (0·02 c.c. blood to a 1 c.c. system), the toxin in a dilution of 1 in 8000 caused haemolysis of human and rabbit blood corpuscles, incomplete after incubation for six hours at 37° C. but complete after a further 18 hours at room temperature. Guinea-pig corpuscles showed only a trace of haemolysis after six hours' incubation using the toxin in a concentration of 1: 500 and no increase in haemolysis had occurred at the end of 24 hours.

In early experiments we used a sample of toxin from Dr. J. W. Trevan, of the Wellcome Laboratories. This, when used, had a lethal dose for mice of 0·14 mg. per 20 gm.

Owing to the rapidity with which this toxin deteriorates in solution in saline we dissolved it in a phosphate buffer solution (M/75 phosphate, pH 7·6) and added 0·01 c.c. of 0·4 p.c. NaOH for each 3·5 mg. of toxin. This brings the pH to approximately 7·4.

Perfusion experiments. The lungs of guinea-pigs were perfused as in the experiments of Feldberg and Kellaway (1937), and the lungs of rabbits and cats with the hilum dependent so as to collect tracheal fluid (Kellaway, Holden and Trethewie, 1938). The perfusion rates varied from 1 to 2 c.c. per minute. The perfusate was tested for histamine and slow-reacting muscle-stimulating substance on the isolated jejunum of the guinea-pig suspended in oxygenated Tyrode solution in a bath of 7 c.c. The histamine estimated is expressed as dichloride. The hind limbs of rabbits and cats were perfused through the aorta by a cannula inserted distal to the renal arteries, the outflow being collected from a cannula in the inferior vena cava. The vessels to the colon and bladder and in the female to the uterus and adnexa were tied off and the muscles of the body wall were ligated "en masse". The spinal vessels were obstructed by plugging the vertebral canal. The venous outflow from the preparation was promoted by placing it in an inclined position reversed during the injection of toxin into the arterial cannula. The liver of rabbits was perfused through the portal vein as described for the liver of the dog (Feldberg and Kellaway, 1938). Adenyl compounds in the perfusate were estimated on the heart of the atropinized guinea-pig by the method of Drury, Lutwak-Mann and Solandt (1938), the cardio-depressant effects being matched with adenosine (B.D.H.). When the perfusate from the liver of the rabbit and from the perfused hind limbs was to be tested for adenyl compounds it was collected, unless otherwise stated, over a boiling water

[1] This work has been carried out under a grant from the National Health and Medical Research Council.

bath. Toxin present in the perfusate was neutralized with excess of anti-toxin before testing. In experiments in which sodium cyanide was added to the perfusion fluid, the procedure was as described by Kellaway and Trethewie (1940a). Extracts of organs for the estimation of histamine and of adenyl compounds were made by grinding weighed amounts with finely divided silica in Tyrode solution (1: 50 or 1: 20) and boiling.

Experiments on the suprarenal glands. The direct and immediate action of the toxin was studied by injection into the cut stump of the coeliac axis of eviscerated cats with the carotid blood pressure recording. The delayed effect was observed by intravenous injection in guinea-pigs in which the whole or part of the right suprarenal gland had been removed to make a control extract. The residual content of the remaining gland was determined at death. Extracts were made by grinding the weighed suprarenal tissue with silica in acid saline (1: 40 to 1: 100). The control extract was stored in the refrigerator until ready for testing. Estimations of adrenalin were made on the carotid blood pressure of the fully pithed cat and results are expressed as chloride in mg. per gm. of suprarenal tissue.

Experiments on plain muscle. Single horns of the uteri of virgin guinea-pigs and rats were suspended in a 7 c.c. bath of oxygenated anaphylactic Ringer's solution (NaCl 0·9 p.c., KCl 0·042 p.c., $CaCl_2$ 0·012 p.c., glucose 0·1 p.c., $NaHCO_3$ 0·05 p.c.). The behaviour of the bronchial smooth muscle was observed by recording changes of intrapleural pressure in artificially-ventilated pithed guinea-pigs. A pleural space was connected with a Brodie's tambour by a glass cannula inserted between the ribs. Changes in pressure in the pulmonary artery of the rabbit were recorded by the method described by Kellaway and LeMessurier (1936).

Perfusion of the hearts of cats and rabbits was carried out as described by Feldberg and Kellaway (1938) using Gunn's modification (1913) of Langendorff's method. The temperature of the perfusing fluid despite variations in coronary flow did not alter more than 0·4° C. The Ringer's solution used for perfusion was maintained at a pH of 7·5 and oxygenated by bubbling oxygen containing 3 p.c. CO_2 through the reservoir. The flow through the coronaries was recorded electrically by a Condon's tipper (1913) with a celluloid bucket.

RESULTS.

The Failure of the Toxin of Cl. welchii *A to Liberate Histamine or S.R.S. from Perfused Organs.*

Experiments were made upon isolated perfused lungs of cats, rabbits and guinea-pigs. After injection into the pulmonary artery of toxin in doses ranging from 30 to 60 mg. no histamine could be detected in the out-flowing perfusate nor was there any significant diminution of the content of histamine in the perfused organ by comparison of extracts of injected and control lung. Furthermore there was no output of a slow-reacting muscle stimulant substance (S.R.S.) like that set free in other types of injury—by snake venom, Feldberg and Kellaway (1938), Feldberg, Holden and Kellaway (1938); by anaesthetics (Kellaway and Trethewie (1939), and by anaphylactic shock, Kellaway and Trethewie (1940b).

(a) *Experiments on guinea-pigs.* The lungs of a guinea-pig weighing 690 gm. were perfused with Tyrode solution through the pulmonary artery at a rate of 1·5 c.c. per minute. The perfusate before the injection of 60 mg. of toxin contained no detectable histamine. The sample of perfusate collected during the first five minutes after the injection had some immediate stimulant effect upon the guinea-pig gut, but a similar effect was obtained by neutralized toxin in the amount estimated to be present in the sample tested, and the response of the gut must be attributed to a histamine-like impurity in the toxin itself. Perfusate collected during a further period of 90 minutes contained no detectable histamine. The perfused organ was now cut down and drained and the drainage fluid (2 c.c.) was found to contain histamine in a concentration of 1: 7 millions. The control lung contained 70γ histamine per gm. and at the end of the experiment the injected lung had a content of 60γ histamine per gm. The lung before injection of the toxin weighed 3·5 gm. and after draining 8·7 gm. After removal of oedema fluid by pressure between filter papers it weighed 4 gm. The slightly lower content per gm. of the lung after injection may be accounted for by failure to remove all oedema.

As it seemed possible that the time allowed for the action of the toxin was insufficient, a lethal dose of toxin was injected into guinea-pigs and after a variable period the lungs were isolated and perfused.

A guinea-pig weighing 485 gm. was killed 18 hours after the subcutaneous injection of 5 mg. of toxin. The lungs were isolated and perfused. The perfusate was free from histamine and the injection of 30 mg. of toxin into the pulmonary artery caused no output of histamine into the perfusate during the next three hours. The control sample of lung and the lung after this further treatment with toxin and subsequent perfusion each contained 18γ per gm. histamine.

A guinea-pig weighing 470 gm. died two hours after the intravenous injection of 2 mg. of toxin. The lungs were at once perfused for 90 minutes at a rate of 1 c.c. per minute and neither histamine nor S.R.S. was detected in the perfusate. A dose of 40 mg. of toxin was now injected into the pulmonary artery. The sample of perfusate collected during the next two minutes had an immediate stimulant action upon the isolated jejunum of the guinea-pig, but a similar effect was obtained with a dose of neutralized toxin equivalent to that present in the amount of sample tested. During the next 105 minutes, the rate of outflow having fallen to 0·5 c.c. per minute, neither histamine nor S.R.S. could be detected in the perfusate nor in and drainage fluid (2·5 c.c.). The lung before injection weighed 1·2 gm. and after drainage 2·9 gm. The control sample of lung contained 14γ of histamine per gm. and the lung after perfusion and injection of toxin 11γ of histamine per gm. of tissue.

In all these experiments the injection of toxin caused broncho-constriction so severe that it became difficult to ventilate the lung, and in the last the lung was already fixed and did not collapse when the thorax was opened immediately after death. Failure to ventilate the perfused lungs adequately may in part account for the oedema observed in them.

These results were confirmed in a further series of experiments. Guinea-pigs were anaesthetized with ether, a tracheal cannula was tied in, and the lungs artificially ventilated. The right side of the thorax was now opened and the diaphragmatic lobe removed and used to make a control extract; the thorax was closed and toxin (1·5 to 2 mg.) injected into a jugular vein. Immediately after death, one to seven hours later, the histamine content of a piece of the left diaphragmatic lobe was determined. The results of 10 experiments are set out in Table 1.

TABLE 1.

Effect of Cl. Welchii A toxin on the histamine content of the lungs of guinea-pigs.

Weight of animal in gm.	Dose of toxin in mg.	Time of death.	Histamine content in γ per gm. Right lung.	Left lung.
515	0·5 (repeated 4 hrs. later)	5 hrs.	23	23
500	1	5 hrs. 15 mins.	6	9
590	1·5	2 hrs. 26 mins.	28	37
590	1·5	3 hrs. 52 mins.	45	56
480	1·5	2 hrs. 50 mins.	25	14
455	1·5	7 hrs.	15	13
630	2	2 hrs. 25 mins.	19	22
570	2	2 hrs. 35 mins.	13	13
520	2	3 hrs.	20	14
480	2	1 hr. 16 mins.	17	17

The content of the right lung was sometimes more and sometimes less than that of the left, but the mean for the right (control) lungs was 21·1γ and for the left 21·8γ per gm. tissue. The histamine contents of the perfused right and left lungs of guinea-pigs do not vary more than 25 p.c. (Feldberg and Kellaway, 1937); in these experiments the greater variation observed may have been related to the difficulty of removing excess of blood from these unperfused tissues before weighing the samples for extraction.

(b) *Experiments on rabbits.* The lung of the rabbit contains but little histamine and is therefore unsuitable for experiments of this kind. Two experiments gave wholly negative results.

The apical lobes of a rabbit weighing 3 kg. were perfused at a rate of 2 c.c. per minute. The perfusate during forty minutes was free from histamine and S.R.S. Toxin in a dose of 40 mg. was now injected into the pulmonary artery. The sample of perfusate collected during the first five minutes after this injection had an immediate stimulant action on the guinea-pig jejunum equivalent to that of 1: 11 million histamine. Perfusate collected during the next three hours and the drainage fluid obtained at the end of the experiment contained neither histamine nor S.R.S. in detectable amounts. The uninjected control lung and the lung after injection of toxin and prolonged perfusion both contained 7γ histamine per gm. of tissue. The perfused lung before injection weighed 5·1 gm. and at the end of the experiment after drainage 5·5 gm. The stimulant activity of the first sample of perfusate was due to an impurity in the toxin present, because neutralized toxin in this amount had a like stimulant effect upon the gut, and because in a control experiment the sample collected during the first five minutes after the injection of 40 mg. of neutralized toxin had a stimulant effect on the gut equivalent to 1: 9 millions histamine. In this control experiment there was no further output of histamine during perfusion for three hours.

(c) *Experiments in cats.* (i) *Isolated perfused lung.* Owing to a severe epidemic of chronic bronchopneumonia it was difficult to obtain healthy lungs for perfusion and we performed only two experiments. In one the perfused lung weighed 5 gm. The perfusate (at 1·5 c.c. per minute) before the injection of toxin contained no detectable amount of histamine or S.R.S.

During two-and-a-half hours after the injection of 40 mg. of toxin neither histamine nor S.R.S. was present in the outflowing perfusate in estimable amounts except in the sample collected during the first five minutes, which possessed histamine-like activity equivalent to 1: 6·6 millions of histamine due to impurity in the toxin. The drainage fluid (2·5 c.c.) contained 1: 9 millions histamine. The lung weighed 8 gm. at the end of the experiment. The control lung contained 25γ and the perfused injected lung 17γ per gm. histamine. In a control experiment in which 40 mg. of neutralized toxin was injected the first sample after the injection also contained histamine-like activity equivalent to 1: 6 millions histamine, and though there was no further output of histamine in the perfusate the drainage fluid contained 1: 11 millions histamine.

(ii) *Isolated hind limb.* When both hind limbs were perfused through the aorta with Tyrode solution at a rate of 1 c.c. per minute no histamine could be detected in the perfusate from the inferior vena cava. After tying off the right iliac artery the rate of flow was adjusted to 1 c.c. per minute and 60 mg. of toxin were injected. The perfusate during three-and-a-half hours contained neither histamine nor S.R.S. The rate slowed to 0·5 c.c. a minute between 15 to 30 minutes after the injection, and towards the end of the second hour fell to 0·16 c.c. per minute. This decrease may be partly due to the vaso-constrictor effects of the toxin, but in the later stages oedema of the limb must have been largely responsible. A piece of sartorius muscle from the right limb and a corresponding piece from the left limb after the experiment each contained 1·5γ histamine per gm. Samples of skin from the right limb contained 21γ histamine per gm. and from the left after perfusion 28γ per gm. In other experiments in which both hind limbs were perfused and 120 mg. of toxin were injected neither histamine nor other muscle stimulant substance could be detected in the perfusate during three hours. Owing to the difficulty of complete removal of subcutaneous tissue from the control skin samples the histamine content was less than in corresponding samples taken at the end of the experiment which, because of oedema, could be more readily freed from connective tissue. In one such experiment the control sample contained 40γ histamine per gm., and that estimated after perfusion with toxin contained 54γ per gm.

The Liberation of Adenyl Compounds and of Inactivating Enzyme by the Toxin of Cl. welchii *A.*

(a) *Experiments on the perfused liver of the rabbit.* The toxin of *Cl. welchii* A, like that of *Cl. welchii* D (Kellaway, Trethewie and Turner, 1940) and the venom of the indian cobra (Kellaway and Trethewie, 1940a) causes the liberation from the perfused liver of the rabbit of adenyl compounds and of an agent capable of inactivating them. Extracts of several pieces from the same liver when tested on the atropinized heart of the guinea-pig by the method of Drury, Lutwak-Mann and Solandt (1938) have the same cardio-depressant effect per gm. of tissue, and prolonged intraportal perfusion with Tyrode solution does not cause any loss of this activity nor can any adenyl compounds be detected in the outflowing perfusate (Kellaway and Trethewie, 1940a). When the toxin of *Cl. welchii* A is injected adenyl compounds can be estimated in the outflowing perfusate within 10 minutes, and the output continues for at least two hours. Pigment appears in the perfusate and the liver becomes reticulated by de-pigmentation which increases throughout the experiment. Increasing amounts of protein precipitated by heat are also observed in the perfusate.

A piece of liver weighing 15·4 gm. from a rabbit weighing 2·95 kg. was perfused at a rate of 2·5 c.c. per minute and during 20 minutes before the injection of toxin no adenyl compounds could be detected in the perfusate. After injection of 60 mg. of toxin the perfusate collected in the first three minutes contained no detectable adenyl compounds. The activity of the samples collected in the next seven minutes and the following 20 minutes was equivalent to 2·4γ and 3·2γ adenosine per c.c. In the next half-hour the perfusate contained the equivalent of 3·0γ adenosine per c.c. and in the second hour 2·5γ adenosine per c.c. The total output of adenyl compounds in two hours' perfusion after the injection was equivalent to 0·71 mg. adenosine. A control piece of liver contained adenyl compounds equivalent to 1·3 mg. adenosine per gm. and the liver which, after the perfusion, weighed 15·1 gm., contained the equivalent of 1·1 mg. adenosine per gm. The loss of adenyl compounds from the perfused lobe as estimated by difference of the activity of these extracts was equivalent to 3·1 mg. adenosine.

In this, as in experiments in which injury was caused to the perfused organ by cobra venom or by the toxin of *Cl. welchii* D, the amount of active cardio-depressant substances estimated in the perfusate was much less than the total loss from the organ estimated by difference of organ extracts. This must be accounted for by inactivation of the adenyl com-

pounds during the perfusion because after the injection of this toxin, as after the injection of cobra venom or the toxin of *Cl. welchii* D, an inactivating agent can be demonstrated in the perfusate if this is collected at room temperature. It is destroyed by heating the perfusate.

Samples of perfusate from a piece of liver before and after intraportal injection of 60 mg. of toxin were collected at room temperature and 1 mg. adenosine was added to 50 c.c. of each making a concentration of 1: 50,000. A portion of each mixture was at once brought to the boil. The two mixtures were then incubated in a water bath at 40° C. for one hour and small amounts were taken from each and boiled after 10, 25, 40 and 60 minutes. The successive tests of the perfusate before the injection of toxin showed no loss of adenosine during incubation. Those of the perfusate after the injection of toxin showed no appreciable loss in 10 minutes, 10 p.c. loss in 25 minutes, 20 p.c. loss in 40 minutes and 40 p.c. loss in one hour.

All these effects of the toxin on the perfused liver (the liberation of coagulable proteins, of pigment, of adenyl compounds and of an inactivating agent) are specific and do not occur when fully neutralized toxin is injected intraportally.

Toxin in a dose of 90 mg. neutralized by admixture with 0·3 c.c. of antitoxin was injected into a lobe of liver weighing 15 gm. No adenyl compounds were detected in the perfusate during perfusion for two-and-a-half hours. The content of the liver in adenyl compounds as estimated in a control extract was equivalent to 0·69 mg. adenosine per gm. and after perfusion and injection of neutralized toxin was equivalent to 0·70 mg. adenosine per gm. The outflowing perfusate was collected partly at room temperature and partly over a boiling water bath. In the latter no adenyl compounds were detected, and when adenosine was mixed with the perfusate collected at room temperature and the mixture incubated for an hour at 40° C. no loss of adenosine occurred.

After the injection of un-neutralized toxin the difference between the output of adenyl compounds estimated in the perfusate and the total loss from the organ cannot be accounted for by action of inactivating enzyme on the perfusate since this was collected over a boiling water bath, but may be due to inactivation taking place within the blood vessels or tissues of the perfused organ. As with cobra venom we have attempted to prevent this loss by perfusing the organ with Tyrode solution to which NaCN was added to make a concentration of M/40. Perfusion with cyanide solution of this strength brought to a pH of 7·5 does not cause any liberation of adenyl compounds into the perfusate, nor is there any significant loss of adenyl compounds from the perfused organ. In Table 2 are shown the results of two experiments in which 90 mg. of toxin were injected in preparations perfused with cyanide Tyrode solution. The first and last experiments in the table are controls. In the first, 90 mg. of toxin were injected into a lobe of liver perfused with Tyrode solution without added cyanide, and in the last the liver was perfused with cyanide Tyrode but no toxin was injected.

TABLE 2.

Liberation of adenyl compounds from the perfused liver of the rabbit by the toxin of Cl. welchii *A in the presence of cyanide.*

Concentration NaCN in the perfusion fluid.	Dose of toxin in mg.	Content of tissue in mg. of adenosine per gm.	P.c. loss by difference of organ extracts.	Output in the perfusate in mg. adenosine.	Output as p.c. of total content of tissue.
Nil	90	0·9	39	0·33	5·6
M/40	90	1·25	14	0·9	6·8
M/40	90	0·92	18	0·96	6·6
M/40	Nil	1·0	3	Nil	0

The effect of cyanide in this concentration was to diminish the total loss of adenyl compounds after the injection of toxin and to increase the output in the perfusate. The action of the toxin is seen to be feeble in comparison with that of cobra venom (Kellaway and Trethewie, 1940a) and even with that of *Cl. welchii* D (Kellaway, Trethewie and Turner, 1940).

(b) *Experiments on the isolated perfused hind limbs of the rabbit.* The hind limbs of a rabbit weighing 2·7 kg. were perfused through the aorta with Tyrode solution (at 4 c.c. per minute) and the perfusate collected from the inferior vena cava over a boiling water bath. During 15 minutes before the injection the perfusate contained no detectable amount of adenyl compounds. Toxin in a dose of 100 mg. was injected into the arterial cannula and was allowed to act for three minutes. Perfusate collected during the first 10 minutes after the injection contained too much toxin for satisfactory estimation of adenyl compounds. The samples collected during the remainder of the first half-hour and during the second half-hour contained in 1 c.c. adenyl compounds equivalent to 1γ and 2γ adenosine respectively. Those collected during the second and third hours contained the equivalent of 2γ and 3γ adenosine per c.c. There was a steady decline in outflow to 0·5 c.c. per minute. The maximum output of adenyl compounds (3·7γ per minute) occurred during the second half-hour after the injection. The output during the last twenty minutes of the first half-hour was 2·8γ and during the second and third hours it was 1·7γ and 1·6γ per minute respectively. The total output

estimated in the perfusate was 0·376 mg. This represented the content of only 1·5 gm. of muscle because a sample of psoas before the injection contained the equivalent of 0·5 mg. adenosine per gm., and at the end of the experiment another sample contained 0·25 mg. per gm. Only a small proportion of the adenyl compounds lost from the muscle were therefore estimated in the perfusate.

The injection of toxin in this preparation also causes the liberation of an agent which inactivates adenosine. The hind limbs from a rabbit weighing 2·3 kg. were perfused with Tyrode solution at 2 c.c. per minute and 150 mg. of toxin were injected. The perfusate was collected at room temperature during half-an-hour before and half-an-hour after the injection, and adenosine was added to these samples to make a concentration of 1: 100,000. Portions of the two mixtures were boiled at once and after incubation for 20, 40, 60, 80 and 100 minutes at 37° C. and their content of adenosine was estimated by comparison with freshly boiled 1: 100,000 adenosine in Tyrode solution. The solution in normal perfusate showed no appreciable loss of activity after incubation for 20 minutes; after 40 minutes there was a slight loss of activity, and after 100 minutes it still retained two-thirds of its original activity. The solution in perfusate after injection of toxin had lost most of its activity after 20 minutes and no detectable activity remained after 40 minutes.

In control experiments with toxin neutralized by antitoxin no trace of adenyl compounds could be detected in the perfusate and there was no significant loss of adenyl compounds from the muscle in the limb.

In a hind limb preparation into which a dose of 150 mg. of toxin mixed with 0·5 c.c. of antitoxin was injected, no adenyl compounds were detected in the perfusate during three hours. The initial rate of flow was 3 c.c. per minute and during the last two hours the rates were 2·5 and 2 c.c. per minute respectively. A sample of muscle before the injection contained the equivalent of 0·3 mg. adenosine per gm., and at the end of the experiment another sample contained 0·25 mg. per gm.

In the hind limb preparation the addition of cyanide to the perfusion fluid had but little effect in preventing the inactivation of adenyl compounds after the injection of toxin. The results even with M/35 cyanide were not significantly different from those obtained when no cyanide was used.

The hind limbs from a rabbit weighing 2·65 kg. were perfused at 5 c.c. per minute with Tyrode containing M/35 NaCN (brought to a pH of 7·5 by the addition of HCl) and 150 mg. of toxin were injected into the aortic cannula. The perfusate before the injection contained no detectable amount of adenyl compounds. No attempt was made to estimate the adenyl compounds in the sample of perfusate collected during the first three minutes after the injection; during the next seven minutes, during the remainder of the first half-hour and during the second half-hour adenyl compounds equivalent to 1·5γ of adenosine per c.c. were estimated. No activity was detected during the second and third hours of perfusion when the rate of flow had fallen to 1 c.c. per minute. The drainage fluid collected during half-an-hour at the end of this period also contained no detectable adenyl compounds. A control sample of muscle contained adenyl compounds equivalent to 0·5 mg. adenosine per gm. and a sample at the end of the experiment contained only 0·3 mg. per gm. The total amount of adenyl compounds estimated in the perfusate was 0·336 mg. or less than that contained in 1 gm. of muscle.

The Liberation of Adrenalin from the Suprarenal Glands.

Using the direct method we failed to demonstrate any immediate output of adrenalin from the suprarenal glands of cats. Intra-arterial injection of 10 mg. of toxin into the cut stump of the coeliac axis in the eviscerated animal failed to cause any greater effect upon the systemic blood pressure than the same dose of neutralized toxin in a control experiment. The effects produced by this large dose must therefore have been caused by histamine or other impurities in the toxin.

The intravenous injection of toxin in a dose of 15 mg. in guinea-pigs weighing from 600 to 660 gm. caused a significant fall in the residual content of adrenalin in the remaining suprarenal, the right adrenal in whole or part having been excised before the injection. The results of two experiments and of a control in which neutralized toxin was injected are set out in Table 3.

TABLE 3.

The delayed effect of Cl. Welchii type A toxin on the suprarenal glands.

Dose of toxin.	Time after injection to death.	Content of suprarenal glands in mg. per gm. Right (Control).	Left
15 mg. neutralized with 0·05 c.c. antitoxin	Killed after 1 hour	0·14	0·14
15 mg.	28 min.	0·17	0·03
5 mg. and 45 min. later 10 mg.	69 min.	0·22	0·11

The observed diminution in the residual content of adrenalin is a specific effect. It cannot, however, be attributed with certainty to a delayed direct action of the toxin since it may be due to splanchnic stimulation.

The Effect of the Toxin of Cl. Welchii *Type A on Smooth Muscle.*

Buttle and Trevan (1928) have shown that this toxin has a strong stimulant action upon the isolated uterus of the virgin rabbit. It has a similar stimulant action upon the isolated jejunum and virgin uterus of the guinea-pig and upon the uterus of the virgin rat. The two samples of toxin used showed slightly different behaviour when tested on the gut and uterus of the guinea-pig but not on the uterus of the rat. The toxin prepared at the Commonwealth Serum Laboratories gave a non-specific immediate response owing to the presence in it of a histamine-like im-

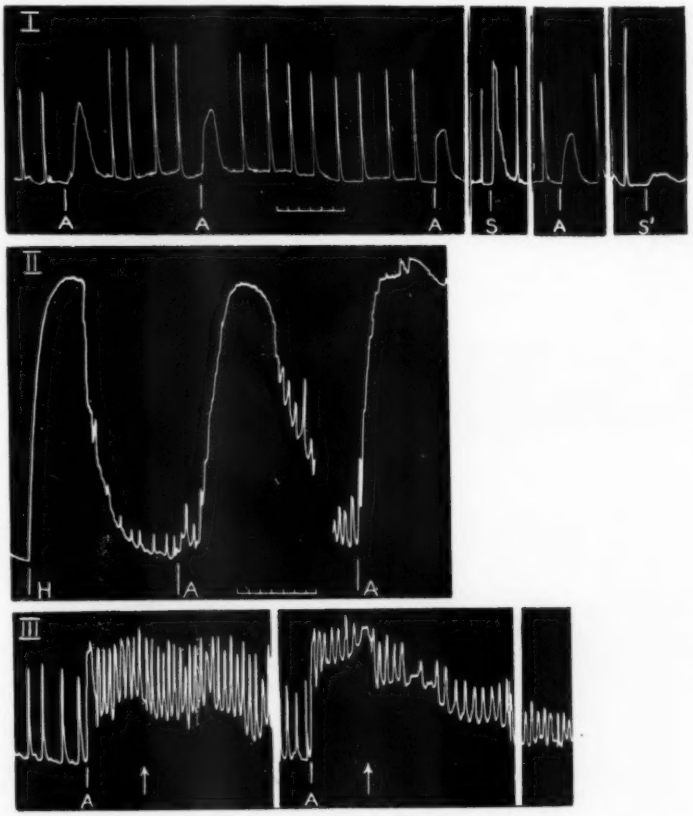


Fig. 1. (I) Record of contractions of two pieces of jejunum from a guinea-pig. At A, 10 mg. toxin, at S, 1 mg. and at S′, 4 mg. of S.R.S. were added to the bath. The unlettered contractions are to 0·1γ histamine.

(II) Record of contractions of a horn of the uterus from a virgin guinea-pig. At H, 2γ histamine and at A, 5 mg. of toxin were added to the bath.

(III) Record of contractions of a horn of the uterus from a virgin rat. At A, 5 mg. A toxin. Interval between the panels 20 and 28 minutes. Time in minutes. Details in text.

purity. The results described for these two preparations were obtained with the Wellcome toxin.

(a) *The isolated jejunum of the guinea-pig.* The toxin in doses of 2 to 10 mg. causes specific contraction of the gut after latent periods of from two minutes to 24 seconds. After repeated doses at short intervals the later responses are greatly diminished in size, but if the interval between the doses is adequate (15 to 20 minutes) their extent is only slightly diminished. The latencies of these contractions are usually shortened (Fig. 1, I). The toxin does not diminish the excitability of the gut as measured by its response to histamine, and may even cause temporary increase of excitability when this has been diminished by other toxins, for example by *Cl. welchii* C, but the response to the slow-reacting muscle-stimulating substance (S.R.S.) formed by the action of snake venom on egg yolk is greatly diminished (Kellaway and Trethewie, 1940b). Fig. 1, I shows the responses (at A) to four successive doses of 10 mg. of toxin which had latencies of 25, 5, 3 and 3 seconds. The later responses were little diminished in size, and those to histamine (0·1γ) were enhanced. The response of another piece of jejunum to 4 mg. S.R.S. (at S′) after four doses of 4 mg. of toxin given during one hour was insignificant though an initial dose of 1 mg. S.R.S. (at S) had given a good response with a latency of five seconds.

(b) *The isolated uterus of the virgin guinea-pig.* The toxin in doses of 0·5 to 5 mg. caused slow contraction of the uterus with latencies of from four to one minutes. Relaxation after the larger doses was slow, taking from half-an-hour to one-and-a-half hours, and depending on the time for which the toxin was left in contact with the muscle. When large doses were given at intervals of half-an-hour or more there was little diminution in the extent of the second or third responses. Sometimes these later responses had shorter latencies. If several small doses were given at intervals of 14 to 18 minutes the later responses were diminished in size and their latencies were slightly increased. For example, a dose of 0·5 mg. gave a moderate response with a latency of three-and-a-half minutes, but the fourth and fifth doses gave smaller stepped responses with latencies of three-and-three-quarters and four minutes. These effects were specific, the neutralized toxin having no action. Fig. 1, II shows the response of one horn of a uterus at H to 2γ histamine and at A to two successive doses of 5 mg. of toxin at 45 minutes interval. These were left in contact with the muscle for seven minutes.

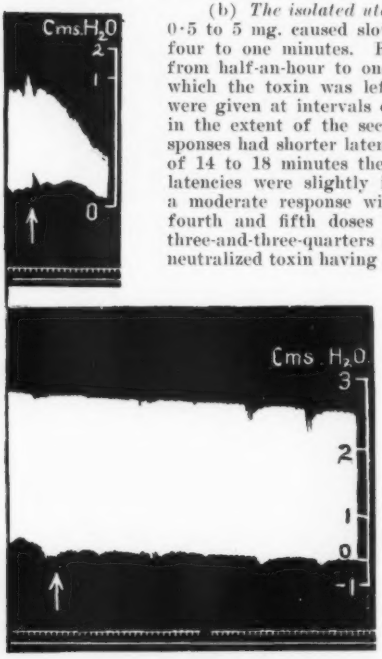


Fig. 2. Records of intrapleural pressure of two pithed guinea-pigs. At the arrow in the upper panel 15 mg. toxin and at that in the lower panel 15 mg. of neutralized toxin injected intravenously. Pressure in cm. of water. Time in half-minutes.

(c) *The isolated uterus of the virgin rat.* The toxin in doses ranging from 2 to 5 mg. caused the muscle to contract, the rhythm at the same time increasing in frequency and diminishing in extent. The contractions in response to second and later doses were usually increased. After several doses the rhythm of the gut was diminished. Fig. 1, III shows the responses to two successive doses of 5 mg. of toxin each left in contact with the muscle for five minutes. Relaxation after the first dose was complete in 36 minutes and after the second in 45 minutes. The rhythm of the uterus after this treatment though increased in frequency was greatly diminished in extent. These effects were specific, the neutralized toxin failing to cause either contraction or change in rhythm.

(d) *The bronchial smooth muscle of the guinea-pig.* When toxin in doses of 5 to 10 mg. is injected subcutaneously into guinea-pigs the lungs after death are found to be distended, and do not collapse when the thorax is opened. Experiments in which the intra-pleural pressure was recorded in pithed guinea-pigs showed that this effect was specific and was due to constriction of the bronchi. The upper panel of Fig. 2 shows the changes in intrapleural pressure in such a preparation after the injection of 15 mg. of toxin into a jugular vein at the arrow. A decrease in the variation of pressure caused by constant artificial ventilation was observed after 90 seconds and in seven minutes the excursion was greatly reduced. The thorax was now opened and the lungs were seen to be distended and did not collapse completely when the artificial ventilation was discontinued. They were free from oedema. The lower panel of the

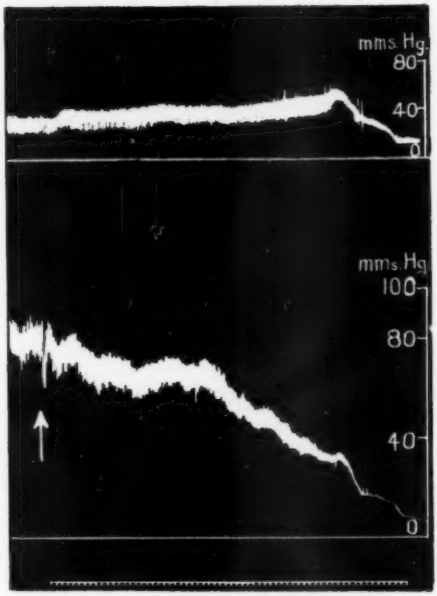


Fig. 3. Record of pressure in the pulmonary artery (upper tracing) and carotid artery (lower tracing) of a rabbit. At the arrow 27 mg. of toxin were injected. Time in half-minutes.

figure shows the record of intra-pleural pressure from another preparation in which a dose of 15 mg. of toxin neutralized by the addition of 0·04 c.c. antitoxin was without effect on the intra-pleural pressure. The slight variations were associated with spontaneous movements. The lungs collapsed completely when the thorax was opened and artificial ventilation discontinued 30 minutes after the injection.

(e) *The pulmonary vessels of the rabbit.* The intravenous injection of toxin in the rabbit causes a gradual rise of pressure in the pulmonary artery which may be attributed to constriction in the pulmonary vessels, and is associated with the development of some oedema of the lung. Fig. 3 shows the changes in blood pressure in the pulmonary and carotid arteries in a rabbit weighing 3 kg. after the intravenous injection of 9 mg. of toxin per kg. During 24 minutes there was a gradual rise in the mean pressure in the pulmonary artery from 16 mm. to 29 mm. Hg, and a fall in the systemic blood pressure from 76 mm. to 32 mm. of Hg. Both pressures then fell sharply as the heart failed. In three experiments the injection of neutralized toxin (18 mg. per kg.) was without effect on the systemic or pulmonary blood pressure, and in no case was there any oedema of the lung.

(f) *The coronary vessels of the rabbit.* We have confirmed the observation of Nicholson (1934) that Cl. welchii A toxin causes constriction of the coronary vessels of the rabbit. When toxin is injected into the perfused coronary vessels of the rabbit's heart there is a gradual falling-off in the rate of flow accompanied by slowing of the heart beat. Fig. 4B shows the effect of 10 mg. of toxin, the outflow falling in the course of one hour from 17·6 c.c.

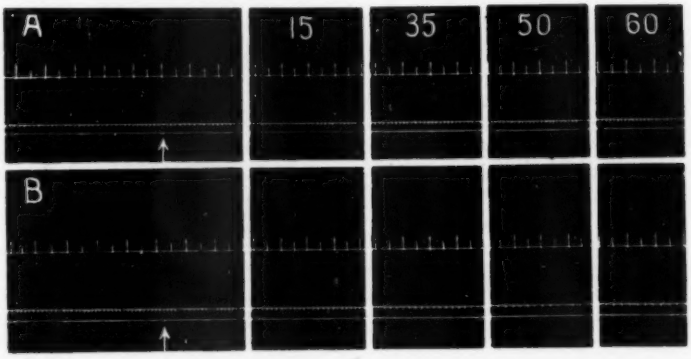


Fig. 4. Records of outflow from the coronary vessels of perfused hearts of rabbits. A. Control. At the arrow 10 mg. neutralized toxin injected. B. At the arrow 10 mg. toxin injected. Upper tracings time in half-minutes. Lower tracings volume records A, 1 division = 2·8 c.c. B, 1 division = 2·4 c.c. The successive records from left to right were obtained 15, 35, 50 and 60 minutes after the injections.

to 8·5 c.c. per minute. The rate of the heart-beat also fell gradually from 172 to 120 per minute. In another experiment after the injection of 15 mg. the outflow fell from 21·5 to 9·1 c.c. per minute and the heart rate from 180 to 65 per minute, block occurring thirty-five minutes after the injection. In control experiments in which no injection was made there was no significant change in the rate of the beat or the coronary outflow. In those in which neutralized toxin was injected there was a slight and persistent increase in the coronary flow from 18·3 to 19·7 c.c. per minute after 10 mg. (Fig. 4A) and from 25 to 28 c.c. after 15 mg. of neutralized toxin.

(g) *The coronary vessels of the cat.* Our experiments on the perfused cat's heart did not give such clear-cut results as those on the rabbit's heart, but the toxin regularly caused a much greater diminution in outflow than was observed in control experiments. After the injection of 10 mg. of toxin the outflow from the heart of a cat weighing 2·1 kg. diminished from 65 c.c. per minute before the injection to 36 c.c. in 15 minutes, to 22 c.c. in 45 minutes and to 18 c.c. 60 minutes after the injection. The heart rate fell from 186 to 156 per minute during the same period.

In control experiments there was generally also some diminution in the outflow and slowing of the heart-beat. For example, after the injection of 10 mg. of neutralized toxin the outflow from the heart of a cat weighing 4·1 kg. diminished from 64 c.c. per minute to 50 c.c. in 15 minutes, to 37 c.c. in 45 minutes and to 32 c.c. in 60 minutes after the injection. The heart rate fell from 148 to 140 per minute during the experiment.

DISCUSSION.

The failure of the toxin of *Cl. welchii* A to cause the liberation of histamine in detectable amounts from perfused organs reasonably rich in this substance, though it causes definite injury to cells as indicated by haemolysis tests and by the liberation from the perfused rabbit's liver of pigment, coagulable protein, adenyl compounds and an agent capable of inactivating them, must be held to suggest either that the liberation of histamine can take place only when injury is moderately severe or that a specific type of injury, which is not inflicted by this toxin, is necessary to cause it. It is true that the toxin of *Cl. welchii* D, which causes liberation both of histamine and of adenyl compounds, is a more potent and rapidly-acting toxin; but the injury to the tissues caused by the toxin of *Cl. welchii* A both in the intact animal, when the poison has some hours in which to operate, and in perfused organs with only brief contact with the tissues, makes it more likely that the liberation of histamine results from a specific type of injury to tissue cells. It will be noted that we have disregarded the presence of histamine in the drainage fluid and occasional small apparent losses from the organs during perfusion. The former is a non-specific effect and the latter is probably accounted for by difficulty in removing oedema fluid.

Though smaller amounts of adenyl compounds are liberated by this toxin than by that of *Cl. welchii* D the amounts set free by similar concentrations of the two toxins are of the same order, and the difference in degree of cellular injury produced by them seems insufficient to account for the liberation of histamine by one toxin and failure to liberate it by the other. It may be recalled that after injury to tissues by volatile anaesthetics, S.R.S. and histamine were liberated, and after injury by gaseous anaesthetics histamine alone was set free (Kellaway and Trethewie, 1939). The difference between the toxins of *Cl. welchii* A and D in respect of their ability to liberate histamine must be regarded as qualitative rather than quantitative, the capacity to liberate histamine being a property of the non-haemolytic ε constituent (Glenny *et alia*, 1933), of which the D toxin used was almost wholly composed, whereas the haemolytic α fraction (constituting A toxin) fails to do so. The experiments on the suprarenals afforded no evidence of an immediate direct liberation of adrenalin and the diminished residual content of the suprarenals at death from poisoning by the toxin may be explained by splanchnic discharges set up by asphyxia during the fatal illness. The failure to liberate histamine from injured cells may be related to the failure to liberate adrenalin directly from the suprarenal glands.

The specific contraction of plain muscle produced by the toxin of *Cl. welchii* A in the bronchial musculature of the guinea-pig, the pulmonary and coronary vessels of the rabbit, and the gut and uterus of the guinea-pig cannot be attributed to histamine, since there is no evidence of its liberation by the toxin from perfused organs. The specific contraction observed in the uterus of the rat which is relaxed by histamine supports this belief. The contraction must be caused either by the direct action of the toxin or by the liberation or formation of some muscle stimulant substance other than histamine. In perfusion experiments we observed no liberation of a muscle-stimulating slow-reacting substance like that set free in anaphylaxis or formed by the action of snake venoms, and must for the present therefore regard the contraction of muscle caused by the toxin as a direct effect.

SUMMARY.

The toxin of *Cl. welchii* type A fails to cause the liberation of histamine or S.R.S. from perfused organs but sets free pigment and coagulable proteins from the perfused liver, and adenyl compounds and an inactivating enzyme from the perfused liver and hind limbs of the rabbit.

The toxin causes no immediate direct liberation of adrenalin from the suprarenals but the residual adrenalin in the glands in guinea-pigs dying from toxaemia is greatly diminished.

In confirmation of other workers, the toxin causes contraction of smooth muscle. This is shown to be a general effect on many kinds of smooth muscle.

These properties of the toxin have been shown to be specific.

REFERENCES.

Buttle, G. A. H. and Trevan, J. W. (1928): Brit. J. exp. path., 9, p. 182.
Condon, N. E. (1913): J. Physiol. Proc., 46, p. 46.
Drury, A. N., Lutwak-Mann, C. and Solandt, O. M. (1938): Quart. J. exp. Physiol., 27, p. 215.
Feldberg, W., Holden, H. F. and Kellaway, C. H. (1938): J. Physiol., 94, p. 232.
Feldberg, W. and Kellaway, C. H. (1937): *Ibid.*, 90, p. 257.
Feldberg, W. and Kellaway, C. H. (1938): *Ibid.*, 94, p. 187.
Glenny, A. T., Barr, M., Llewellyn-Jones, M., Dalling, T. and Ross, H. E. (1933): J. Path. Bact., 37, p. 53.
Gunn, J. A. (1913): J. Physiol., 46, p. 506.
Kellaway, C. H., Holden, H. F. and Trethewie, E. R. (1938): Austral. J. exp. Biol., 16, p. 331.
Kellaway, C. H. and LeMessurier, D. H. (1936): *Ibid.*, 14, p. 57.
Kellaway, C. H. and Trethewie, E. R. (1939): *Ibid.*, 17, p. 225.
Kellaway, C. H. and Trethewie, E. R. (1940a): *Ibid.*, 18, p. 63.
Kellaway, C. H. and Trethewie, E. R. (1940b): Quart. J. exp. Physiol., 30, p. 121.
Kellaway, C. H., Trethewie, E. R. and Turner, A. W. (1940): Austral. J. exp. Biol., 18, p. 253.
Nicholson, J. A. (1934): Rep. Dir. Inst. Anim. Path., Cambridge, 4, p. 212.

THE THIOCHROME METHOD OF ESTIMATING THIAMIN (VITAMIN B_1) IN URINE, MILK AND CEREAL PRODUCTS

by E. C. SLATER

(From the Australian Institute of Anatomy, Canberra).

(Accepted for publication 18th December, 1940.)

Several investigators (e.g. Westenbrink and Goudsmit, 1937; Karrer and Kubli, 1937; Ritsert, 1938) have adapted the thiochrome method of Jansen (1936) to the measurement of thiamin in urine. Wang and Harris (1939), after a thorough examination of possible sources of error, recommended an improved and simplified visual method. Thiamin added to urine was recovered quantitatively and excellent agreement was obtained between the chemical method and the bradycardia method. The procedure has been used in an extensive study by Wang and Yudkin (1940). Hills (1939), however, criticizes the Wang-Harris method and claims that quantitative recovery of thiamin added to urine cannot be obtained.

Estimation in urine. In this investigation the method of Wang and Harris was followed with minor modifications. These authors recommended that the amount of ferricyanide added should be carefully controlled and determined in a separate experiment. They state that an excess of ferricyanide interferes by destroying some of the thiochrome and also by having a variable effect on the non-specific fluorescing substances. However in the present study exactly the same fluorescence was obtained with 0·05, 0·20, 0·50 ml. 2 p.c. ferricyanide respectively so the separate control test was omitted and the same concentration of oxidizing agent (0·20 ml.) used throughout.

All results described in this paper have been obtained from urines giving blanks showing only a small fluorescence, and giving good matches.

The wet isobutanol solutions were dried with ethanol. After the volumes were made up to 15 ml., the solutions were kept in a corked test tube and in diffuse light until measurement of the fluorescence (cf. Wiegand (1938)). The thiamin solution (2 micrograms/ml.) used for the preparation of the standard thiochrome solution was unchanged after several months, but it is necessary to prepare a fresh standard thiochrome solution daily. It was found convenient to dilute concentrated urines or those rich in thiamin so that 1·0 to 1·5 ml. of the standard thiochrome solution was required for a match.

Twenty-four-hour samples of urine were collected in bottles containing 5 ml. 10 p.c. HCl and 5 ml. toluene. The volume was measured so that the daily excretion of thiamin could be calculated.

Table 1 shows the recovery of thiamin added to urine.

TABLE 1.

Recovery of thiamin added to urine.

Specimen of urine.	Thiamin in urine micrograms/ml.	Thiamin added micrograms/ml.	Added thiamin recovered micrograms/ml.	Percentage recovery.
D.P.1	0·19	0·18	0·15	83
	0·17	0·34	0·32	94
	0·16	0·45	0·46	102
E.M.D.4	0·17	0·18	0·18	100
	0·15	0·40	0·36	90
T.B.M.1	0·11	0·25	0·29	116
	0·09	0·39	0·44	112
				Mean 100

Otto and Rühmekorb (1938) state that it is necessary to carry out the comparison of the fluorescence quickly as thiochrome is rapidly destroyed in ultra-violet light. However, under the conditions of the above method only 10 p.c. was destroyed after 30 minutes exposure to ultra-violet light. Normally the tubes are kept under the ultra-violet light for only a few minutes.

Estimation in Milk.

The Wang-Harris technique, with minor variations, has been applied to milk for the estimation of both "free" and total thiamin. The total thiamin was measured after a preliminary incubation with takadiastase to convert the bound thiamin to the "free" form (Houston and Kon (1939a)). When slightly acidified milk is shaken with isobutanol and centrifuged, a gelatinous precipitate separates at the junction of the two layers. Accordingly the centrifuged mixture was filtered. Pyke (1939) prefers not to filter because thiamin is adsorbed. However it was found that (1) the same result was obtained with filtration as without (special precautions being

taken to measure the volume of the water layer); (2) quantitative recovery of added thiamin was obtained (see Table 2); (3) the precipitate had not adsorbed any thiamin. Filtering an aqueous thiamin solution containing 0·1 γ per ml. caused a loss of 50 p.c. of the thiamin due to adsorption.

Neuweiler (1938) found it necessary to remove the fat and protein before adsorption on franconite. Probably the fat and protein in milk prevent a similar adsorption.

TABLE 2.

Recovery of thiamin added to milk.

Type of milk.	Thiamin content micrograms/ml.	Added thiamin micrograms/ml.	Recovered thiamin micrograms/ml.	Percentage recovery.
Cow	0·20	0·10	0·12	120
	0·18	0·20	0·25	125
	0·25	0·20	0·23	115
	0·23	0·30	0·33	115
	0·15	0·40	0·39	98
	0·23	0·80	0·94	117
	0·25	1·00	1·03	103
Human	0·11	0·10	0·115	115
	0·11	0·16	0·16	100
				Mean 112

Houston and Kon (1939b) and Pyke (1939) used pepsin and takadiastase to convert bound thiamin to the free form. But the pepsin has been omitted here as it caused a cloudiness in the fluorescence of the oxidized solution. A comparison of the two methods on a milk containing only 34 p.c. free thiamin showed no significant difference. Takadiastase contains about 6 γ thiamin per gm. A neutral solution loses this thiamin on standing one week. If freshly prepared takadiastase solution is used, a correction for this thiamin should be applied.

Experimental method. Two 3 ml. aliquots were pipetted into two 15 ml. centrifuge tubes. To each, 10 p.c. HCl was added to bring the milk to a pH 3·7–4·0 (2 drops 10 p.c. HCl were usually necessary). To one (total thiamin) 1 c.c. 0·5 p.c. takadiastase was added and the mixture incubated at 37° C. for three hours. Each centrifuge tube was then treated identically as follows. The contents were shaken with 5–6 ml. isobutanol, centrifuged and filtered into a graduated test tube. The precipitate was washed with water until the water layer in the filtrate read 6 ml. The filtrate was poured carefully backwards and forwards into another test tube to make the water layer homogeneous and 2 ml. of the water layer taken for the determination of the thiamin. Thereafter the procedure as described for urine was used, except that after shaking with isobutanol and also after washing the isobutanol layer with water it was necessary to separate the layers by centrifuging. 0·2 ml. 2 p.c. potassium ferricyanide was used for the oxidation.

TABLE 3.

Thiamin content of cow's milk.

Type of milk.	Thiamin micrograms/ml. "Free".	Total.	Free/total.
Retail: raw	0·19	0·27	·75
Cow A	0·18	0·20	·90
B	0·21	0·21	1·00
C	0·43	0·43	1·00
D	—	0·93	—
Malted milk A*	3·7†	3·7†	1·00
B	2·1†	2·5†	0·84
Powdered whole milk	1·9†	2·7†	0·69
Reconstituted powdered whole milk	0·27	0·39	0·69

* fortified with vitamin B. † per gm.

Powdered milks and malted milks were reconstituted and determined in the same way. The blanks from malted milks gave a green-yellow fluorescence which was apparently destroyed on oxidation as the oxidized solution showed a good blue-purple fluorescence. As the blank contained very little blue fluorescence and it was impossible to obtain a match with it, a blank from cow's milk was used for the determination of the malted milks. The results are tabulated in Table 3. Those given for retail milk are the means of nine samples from February to June, 1940. The "free" thiamin ranged from 0·16 to 0·24 γ /ml. and the total from 0·19 to 0·34 γ /ml.

Estimation of Thiamin in Cereal Products.

For the estimation in cereal products a known weight of the finely ground substance was weighed into a 15 ml. centrifuge tube, 5 ml. of a buffer solution of pH 3·8 (sodium acetate-acetic acid) and 1 ml. of 1 p.c. takadiastase added and the contents of the tube well shaken. The mixture

was incubated at 37° C. overnight (incubation of pure thiamin under these conditions showed no destruction of the vitamin), shaken with 6 ml. isobutanol and centrifuged. A measured volume of the water layer was taken for the determination of the thiamin in the usual way. It was usually necessary to centrifuge after shaking. The following weights and volumes of the water layer taken for the determination of the thiamin were found most convenient, viz.: white flour 0·4 gm., 2 ml. respectively; wheat germ 0·03 to 0·05 gm., 1 ml.; whole wheat, wholemeal bread (dried), wholemeal flour, 0·2 to 0·3 gm., 1 ml.

(1) *White flour.* The reproducibility of the results using different weights of white flour is shown in Table 4.

TABLE 4.

Reproducibility of results for white flour.

Weight of flour taken (gm.).	Volume standard thiocrome solution required (ml.).	Thiamin microgram/gm.
0·211	0·65	1·23
0·247	0·77	1·25
0·295	0·95	1·29
0·388	1·3	1·34
0·500	1·5	1·20
0·499	1·5	1·20
		Mean 1·25
		$\epsilon = 0·025$

The recovery of thiamin added (in the form of a solution) to white flour is shown in Table 5.

TABLE 5.

Recovery of thiamin added to white flour (1·25 micrograms/gm.).

Added thiamin γ /gm.	Recovered thiamin γ /gm.	Percentage recovery.
1·32	1·25	95
2·16	2·00	92
2·15	1·85	86
2·80	2·46	88
3·38	3·65	108
		Mean 94

(2) *Wheat germ.* Nine results for the same sample ranged from 20·5 to 27·0 with a mean of 24·8 and a standard error of 1·05. Another sample (from the same manufacturer) gave five results ranging from 21·4 to 24·2 with a mean of 22·6 and a standard error of 0·46. The recovery of added thiamin is shown in Table 6.

TABLE 6.

Recovery of thiamin added to wheat germ (22·6 micrograms/gm.).

Added thiamin γ /gm.	Recovered thiamin γ /gm.	Percentage recovery.
16·3	13·4	82
27·2	25·1	92
32·6	29·2	90
		Mean 88

A well known imported wheat germ product assayed at 36 γ /gm. This compares well with the figures published for this product, viz.: 31–43 γ /gm. (Graves (1940)—bradycardia method). To test further that this method extracts the thiamin completely from the wheat germ, 0·06 gm. was incubated as usual with the buffer solution and takadiastase. The residue was filtered off, washed and incubated once again with the buffer solution and takadiastase. The resultant solution contained no thiamin.

A determination without any takadiastase showed that 91 p.c. of the thiamin in wheat germ is in the "free" form (cf. Melnick and Field (1939), who found 89 p.c. in the free form).

(3) *Whole wheat berry.* Eight results for the same sample ranged between 4·10 and 5·10 with a mean of 4·52 and a standard error of 0·16. The recovery of thiamin is shown in Table 7.

TABLE 7.

Recovery of thiamin added to whole wheat berry (4·52 micrograms/gm.).

Added thiamin γ /gm.	Recovered thiamin γ /gm.	Percentage recovery.
4·11	3·83	93
6·05	6·99	115
9·17	10·00	109
9·21	10·06	109
		Mean 106

Wholemeal bread contains 30–36 p.c. moisture which was removed by cutting the bread into thin slices and allowing to stand at atmospheric temperature for about a week. The dried material was then finely ground and the thiamin determined as for whole wheat berry.

The present method was compared with that of Pyke (1939). Determinations were carried out on the same substance using both the method described in this paper and that described by Pyke (1939). The results are shown in Table 8.

TABLE 8.

Comparison between the present method and that of Pyke for cereal products.

	Thiamin γ gm.	
	Pyke's method.	Present method.
Wheat germ	26	25
Whole wheat berry	3·9	4·5
White flour	1·35	1·25

Good matches were obtained by both methods, but a more purple fluorescence was given by the method described in this paper.

The blanks from all the cereal products were entirely free of any blue fluorescence and good matches were obtained. The white flours gave the best matches. In the case of the "germ" breads examined, the blanks showed a greenish fluorescence, but with care a satisfactory match could be obtained. Using reagent blanks prepared from water, the same values were obtained as with blanks from the bread solutions.

Various cereal products have been estimated by this method and a summary is given in Table 9.

TABLE 9.

Results obtained with cereal products.

		Thiamin γ gm.	
Cereal products.	No. of samples analysed.	Range.	Mean.
Whole wheat	17	4·1 –8·6	5·9
Wholemeal bread	2	2·5 –2·5	2·5
"Germ" bread	2	2·0 –2·1	2·0
White flour	15	1·25–2·53	1·75
Bran	1	—	7·4
Pollard	1	—	12·7
Wheat germ: raw	4	25–32	27
Wheat germ: treated commercially	6	13–36	21

These results are in good agreement with those published overseas by the rat growth and bradycardia methods. (Baker and Wright (1938), Harris and Leong (1937), Copping and Roscoe (1937)).

SUMMARY.

The thiochrome method of Wang and Harris can be satisfactorily applied to the determination of thiamin in milk and cereal products. The method described in this paper does not differ appreciably from that of Pyke (1939), but minor alterations in the technique were found desirable. In particular, incubation with takadiastase alone without previous peptic digestion gives a more purple fluorescence, especially in the case of milk. Criticisms by Hills (1939) of the Wang and Harris method of determining thiamin in urine appear to be unjustified.

The reproducibility of the method depends upon the accuracy of the final matching which is about 10 p.c. to 15 p.c. The recovery of added thiamin is quantitative in all cases.

REFERENCES.

Baker, A. Z. and Wright, M. D. (1938): Biochem. J., 32, p. 2156.
Copping, A. M. and Roscoe, M. H. (1937): *Ibid.*, 31, p. 1879.
Graves, H. C. H. (1940): Med. J. Austral., 1, p. 846.
Harris, L. J. and Leong, P. C. (1937): J. Soc. Chem. Ind., 56, p. 195T.
Hills, G. M. (1939): Biochem. J., 33, p. 1966.
Houston, J. and Kon, S. K. (1939a): Nature, 143, p. 558.
Houston, J. and Kon, S. K. (1939b): Biochem. J., 33, p. 1655.
Jansen, B. C. P. (1936): Rec. trav. chim. Pays-bas., 55, p. 1046.
Karrer, W. and Kubli, U. (1937): Helv. Chim. Acta, 20, p. 369.
Melnick, D. and Field, H. (1939): J. biol. Chem., 127, p. 531.
Neuweiler, W. (1938): Klin. Woch., 17, p. 296.
Otto, H. and Rühmekorb, F. (1938): *Ibid.*, 17, p. 1246.
Pyke, M. A. (1939): J. Soc. Chem. Ind., 58, p. 338T.
Ritsert, K. (1938): Deutsch. med. Woch., 64, p. 481.
Wang, Y. L. and Harris, L. J. (1939): Biochem. J., 33, p. 1356.
Wang, Y. L. and Yudkin, J. (1940): *Ibid.*, 34, p. 343.
Westenbrink, H. G. K. and Goudsmit, J. (1937): Rec. trav. chim. Pays-bas., 56, p. 803.
Wiegaud, J. A. (1938): Arch. Neerland. Physiol., 33, p. 312.

INTRANASAL INFECTION OF MICE WITH THE VIRUS OF PSITTACOSIS

by G. V. RUDD AND F. M. BURNET[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Accepted for publication 11th September, 1940.)

In work with the virus of psittacosis, the mouse has been largely used as an experimental animal; the standard route of inoculation has been intraperitoneal, but recently Mauer (1938) has found that pulmonary lesions invariably follow administration of virus intranasally. Hornus (1939, 1940) has described the macroscopic and microscopic appearances of the infected mouse-lung. We have confirmed these results, and now report further investigations in which the intranasal method of inoculation was used. It appears that, when limiting infective dilutions of virus are approached, lesions take the form of discrete foci of consolidation scattered through the lungs. These foci can readily be counted; the counts provide an accurate method of titration of the virus. By this method investigations have been made on immunity to psittacosis and on the effect of the sulphanilamide group of drugs on the course of pulmonary psittacosis in mice.

Materials used. Egg-passage psittacosis virus known to have retained high pathogenicity for mice was administered in high concentration by intranasal instillation; the method was that used in routine work with influenza virus. The lungs were removed from the mice when they became moribund, usually on the second or third day. The lungs from a mouse were ground with quartz powder, taken up in 5 c.c. of broth and lightly centrifuged. Serial dilutions of the supernatant fluid were prepared in ice-cold saline containing 10 p.c. normal horse-serum; these were administered in doses of 0·05 c.c.

The mice used were eight weeks old. Surviving animals were killed for examination eight days after inoculation except where indicated.

The pulmonary lesions. Psittacosis in the mouse-lung gives rise to areas of grey consolidation involving part or the whole of a lobe. Even with large infecting doses of the virus, consolidation, although widespread, is usually not lobar. Often portion of a lobe escapes consolidation and is macroscopically normal and air-containing. Discrete foci of consolidation observed with limiting infective dilutions of virus-suspension have the same grey appearance as the larger areas of consolidation provoked with higher concentrations. They are grey, almost translucent areas 1–3 mm. in diameter and lie in apparently normal lung. Rarely, general congestion of the lungs and fusion of the foci make counting impossible. Focal counts are made either by direct inspection of the lung surfaces or by gently compressing the lobes between glass slides and examining by transmitted light. The counts by these two methods agree, so that the foci of consolidation in almost every instance must reach the surface of the lung at some point.

It is improbable that retrogression of foci of consolidation ever occurs. In one experiment a large group of mice was inoculated with virus diluted 1: 10^6; some examined at 8 and 16 days showed 1, 2, 1, 1, 0, 4, 1 foci. The remainder were left for 35 days; 6 specific deaths occurred from 19–31 days and 5 animals survived. The appearances in the mice dying specifically indicated that the foci enlarged centrifugally but might also produce terminal lobar consolidation. The survivors were used in an immunity experiment described later.

Foci of consolidation in lungs removed eight days after intranasal instillation have been examined microscopically. The foci are sharply demarcated from the surrounding normal lung and consist of an interstitial and alveolar accumulation of mononuclear cells and polymorphonuclear leucocytes, the former predominating. Vascular congestion is present but very few extravascular red cells are to be found. In older lesions, the same appearances are found; often the vascular congestion is great at the periphery of the focus. Elementary bodies (Levinthal-Coles-Lillie bodies), staining blue with haematoxylin and eosin, are always visible in sections of infected lungs removed eight days after inoculation. They are found in mononuclear and occasionally in polymorphononuclear leucocytes, and in the epithelium of the bronchioles.

Smears made from infected lung stained by Castaneda's method show numerous L.C.L. bodies, both intracellular and extracellular. L.C.L. bodies are much less numerous in smears made from lungs in which infection has been established for two weeks or more; great difficulty may be encountered in finding them in old lesions.

Titration of psittacosis virus in the mouse-lung. The accuracy of focal counts in titrating

[1] This work has been carried out under a grant from the National Health and Medical Research Council.

virus was examined. Dilutions of a virus-suspension were made in the usual way; dilutions of 1: 10^7, 10^6 and 10^5 were used as intranasal inocula in three groups of twenty mice. Two non-specific deaths occurred in the 10^7 group, one in the 10^6 group and one in the 10^5 group. Here, typical lung lesions were not observed, nor were L.C.L. bodies present in smears from the lungs.

TABLE 1.

Numbers of foci produced in mice inoculated intranasally with high dilutions of psittacosis virus.

Virus dilution.		Frequency distribution.									Mean.
1: 10^7	Foci:	0	1								0·28
	Number of mice:	13	5								
1: 10^6	Foci:	0	1	2	3	4	5	6	7	12	3·6
	Number of mice:	1	4	1	3	6	1	1	1	1	
1: 10^5	Foci:	7	22	24	25	26	27	29	30	31	38	26·7
	Number of mice:	1	1	3	1	2	2	2	3	2	1	

One mouse in the 10^5 group showed coalescence of the foci, so that an accurate count was not possible. All these mice have been excluded from Table 1 which shows the frequency distribution of the foci in each group. For the eighteen mice considered in the 10^5 group, the standard deviation of the focal counts is 6·0, the coefficient of variation is 22·5 p.c. and the standard error of the mean is 1·43. Therefore the mean of 26·7 is unlikely to differ from the true mean by more than $2 \times 1{\cdot}4 = 2{\cdot}86$, if 18 mice are used in the test-group. In routine titrations of the virus it is impracticable to use such large numbers of mice. With smaller numbers in each group, however, the focal counts follow a similar trend. This is illustrated in Table 2A which records the titration of a virus-suspension prepared from a chorioallantoic membrane, five mice being used in each group. It will be seen in Tables 1 and 2A that when tenfold dilutions of the virus-suspension are used, the means of the focal counts for each of two successive dilutions differ by a factor less than ten. This result has very frequently been observed not only in the titration of the virus of psittacosis in the mouse-lung but also in the titration of viruses on the chorioallantois and in accurate work with plant viruses and bacteriophages.

Table 2 shows the results obtained in a comparative titration of a psittacosis virus suspension by both intranasal and chorioallantoic methods. It will be seen that, in addition to some uncertainty of the counts on the egg membranes indicated by the sign ± in Table 2B, approximately one in fifty of the number of virus particles which established themselves in the mouse-lung succeeded in doing so on the chorioallantois.

TABLE 2.

Titration of psittacosis virus suspension.

A. *By intranasal inoculation*:

Virus dilution.	Foci in lungs.					Mean.
1: 10^7	0	0	1	1	1	0·6
1: 10^6	3	4	4	9	10	6
1: 10^5	27	33	35	38	48	36

B. *By the chorioallantoic method*:

Virus dilution.	Foci on chorioallantois.				Mean.
1: 10^6	0	0	0	0	0
1: 10^5	0	0	2	4	1·5
1: 10^4	0	4	10±	10±	6

In comparing the intraperitoneal with the intranasal method of titration we found considerable difficulty in obtaining a definite end-point by the former method. With the strain used, death of the animal within 14 days with specific lesions resulted only with moderately large doses. With limiting infective dilutions it was often difficult to be sure whether minimal lesions e.g. enlarged spleen and some thickening of the peritoneal folds around the liver were or were not specific. Our impression is that the limiting infective dose was approximately the same by both methods but there was no question as to the much greater definiteness of the intranasal results.

Immunity to Psittacosis.

Using as a test of immunity the intranasal inoculation of high dilutions of virus, attempts were made to immunize mice by the following methods: 1. Repeated intraperitoneal inoculation of virus killed by formalin. 2. Subcutaneous inoculation of living virus. 3. Intranasal inoculation of minimal infecting amounts of living virus.

1. *Immunity after Intra-peritoneal Inoculation of Formolized Virus.* To virus-suspensions prepared in the usual way from mouse-lungs, formalin (40 p.c. HCHO) was added in the proportion 0·5 c.c. to 100 c.c. of suspension. After ten days in the refrigerator this material was used

to inoculate mice intraperitoneally, the dose being 0·5 c.c. Three doses were given at weekly intervals; two weeks after the third dose the mice were given a test dose of living virus intranasally. Two separate series were used, one of which received egg-membrane virus, the other mouse-lung virus. The control mice of both series were of the same age as the immunized. In neither series was there any evidence of immunity when the mice were examined eight days later. The results of the test with egg-membrane virus are recorded in Table 3. Some of the same batch of immunized mice, and controls of the same age, were given intraperitoneal inoculations of living virus. In the control series a proportion of specific deaths occurred at all dilutions up to 1: 10^4, but in the immunized only with the dilution 1: 10. This evidence of partial immunity, when the test dose is given by the intraperitoneal route, confirms the results obtained by Bedson (1933).

TABLE 3.

Test of mice immunized by intraperitoneal inoculation of formolized virus.

Test virus dilution.	Foci in lungs. Immunized mice.				Control mice.		
1: 10^6	0	1	1	2	0	1	2
1: 10^5	3	9	11	11	12	10	x
1: 10^4	72	78	81	95	102	x	x

x = nonspecific deaths.

2. *Immunity after Subcutaneous Inoculation of Living Virus.* Twelve mice were inoculated subcutaneously with 0·1 c.c. of undiluted living virus-suspension. Eighteen days later the inoculations were repeated. After a further fourteen days the mice were divided into groups of three, and intranasal instillations of the same strain ("B") of virus and of virus from another source (strain "P") were made in ten-fold and thousand-fold dilutions. Control mice of the same age received intranasally the same virus-dilutions. The results recorded in Table 4 show no indication of immunity. A similar result was obtained with a small group of four mice which received three immunizing doses subcutaneously over a period of thirty days.

TABLE 4.

Absence of immunity after subcutaneous inoculation as tested against two strains given intranasally.

Test virus dilution.	Lung consolidation. Strain B. Immunes.			Strain B. Controls.			Strain P. Immunes.			Strain P. Controls.		
1: 10^3	**3**	**3**	**4**^{6}	**3**	**3**	**3**	**2**	**2**	**3**	**2**	**3**	**3**
1: 10	**4**^{3}	**4**^{3}	**4**^{5}	**4**^{3}	**4**^{3}	**4**^{4}	**4**^{4}	**4**^{4}	**4**^{5}	**4**^{5}	**4**^{5}	**4**^{6}

All mice showed areas of consolidation which are recorded according to the convention used for influenza virus lesions, 1–4 representing increasing degrees of consolidation, 4 being complete or almost complete, with death on the day shown by the index figure.

3. *Immunity after Intranasal Inoculation.*

(a) *With living virus at limiting dilutions.* Groups of twenty mice were given by intranasal instillation virus at dilutions 1: 10^7, 10^6 and 10^5. Examination of mice killed at eight and sixteen days indicated that approximately 0·3, 3 and 30 minimal infecting doses had been administered. Deaths, most of which were specific, occurred in the 10^5 and 10^6 groups up to the 35th day. At this stage all the survivors and control mice of the same age were given test intranasal inoculations of virus. Only one mouse showed any evidence of immunity; when it was killed 14 days after the test inoculation (10^4 dilution of virus) the lung was normal except for a single area of grey consolidation presumably resulting from the preliminary infection with virus diluted 10^6. All the other mice receiving this test dose both controls and immunes died with specific lesions. There is therefore no evidence of immunity of the mice in which a pulmonary infection had been established for thirty-five days.

TABLE 5.

Absence of immunity after intranasal administration of killed virus.

Test virus dilution.	Lung lesions. Immunized mice.				Control mice.		
1: 10^5	15	29	35	43	36	39	47
1: 10^4	**2**	**3**	**3**	**3**	**1**	**3**	**3**
1: 10^2	**3**	**4**^{5}	**4**^{5}	**4**^{6}	**4**^{5}	**4**^{5}	**4**^{5}

Figures in heavy type refer to degrees of consolidation.

(b) *With formolized virus.* To a virus-suspension prepared from mouse-lungs in the usual way formalin was added to a concentration of approximately 0·2 p.c. formaldehyde. After

incubation overnight, the suspension was given in 0·05 c.c. doses intranasally to twelve mice. Eight days later a second dose was given. Twenty-one days later dilutions of living virus-suspension were instilled intranasally. The results, summarized in Table 5, give no evidence that formalin-killed virus administered intranasally can evoke immunity against infection by the same route.

Presence of neutralizing antibody in immune sera. Serum from a patient convalescent from psittacosis was tested for the presence of neutralizing antibodies. Dilutions to 1: 10^6 and 10^5 of virus-suspension were mixed with an equal volume of undiluted serum and allowed to stand 1½ hours in the refrigerator before intranasal instillation in mice. Normal human serum was used in a similar manner as a control. Examination of the mouse-lungs eight days later revealed that no neutralization of virus-activity had occurred. The results are given in Table 6. Similar negative results were obtained in tests with another human convalescent serum, even when the virus-strain used was that actually isolated from the patient's sputum.

TABLE 6.

Absence of neutralizing antibody in convalescent human serum.

Virus dilution.	Foci in lungs of mice given: Virus + convalescent serum.						Virus + normal serum.					
1: 10^6	0	0	0	1	1	2	1	2	3	3	5	7
1: 10^5	9	16	16	24	24	26	13	13	19	23	44	2

Guinea-pig immune sera were prepared. Two guinea-pigs (198–199) received three intraperitoneal injections of living virus suspensions over a period of six weeks. Two weeks after the last injection, the guinea-pigs were bled and the serum separated. Tests for the presence of neutralizing antibody were made as before; none was found. Table 7 records the results.

TABLE 7.

Test of immune guinea-pig sera for neutralizing antibody.

Virus dilution.	Foci in lungs of mice given virus dilutions plus: Serum 198.					Serum 199.					Normal serum.				
1: 10^6	0	0	0	1	2	0	0	0	1	N–sp.	0	0	0	1	1
1: 10^5	1	1	1	2	3	0	0	0	1	2	0	0	0	1	2
1: 10^4	4	4	13	2		2	6	9	10	10	4	5	9	10	11

Chemotherapy in pulmonary psittacosis in mice. The effect of sulphapyridine on the course of psittacosis infections in the mouse-lung was first examined. The drug was administered by gastric intubation in 20 mg. doses for 10 doses spread approximately equally over a period of seventy-two hours. Virus dilutions were instilled intranasally two hours after the first dose of sulphapyridine. The control mice received no chemotherapy. The results in Table 8 demonstrate no therapeutic effect of sulphapyridine.

TABLE 8.

Failure of treatment with sulphapyridine to influence pulmonary infection with psittacosis virus.

Test virus dilution.	Foci in lungs. Treated with sulphapyridine.					Controls.			
1: 10^5						0	3	6	7
1: 10^4	24	53	54	58		41	78	3	
1: 10^3	3	3	3	3	3	3	3	3	3

A further test was made in order to determine whether somewhat earlier administration of drugs of this series, combined with higher dosage given in part subcutaneously, influenced the pulmonary infection. Sulphapyridine, sulphanilamide, prontosil and proseptasine were administered respectively to four groups of mice. The first dose, of 50 mg., was injected subcutaneously in the groin. Three subsequent doses of 20 mg. were given by gastric intubation. The final (fifth) dose of 50 mg. was injected subcutaneously. The doses were given over a total period of thirty hours. Each group of mice receiving a given drug was divided into three subgroups for intranasal instillation of 1: 10^5, 10^4 and 10^2 dilutions of virus suspension, four hours after the first (subcutaneous) dose of the drug. As in the earlier test with sulphapyridine, counts of foci of consolidation, or evaluation of areas of consolidation in the case of lower dilutions of virus, eight days after infection, revealed no difference between the mice which had received chemotherapeutic drugs and those which had not.

A final test was made to observe the effect of sulphapyridine on the course of infection in mice previously given "immunizing" doses of formolized virus intranasally. The times and methods of the administration of the drug, and of the intranasal instillation of living virus, followed the plan of the preceding test. The attempted immunization with formolized virus intranasally has been described above. Control mice had been given the course of immunization but no chemotherapy and, in addition, the virus suspension was titrated in normal mice by

intranasal instillation. It has been shown above that attempts either to immunize mice by intranasal formolized virus or to alleviate the course of psittacosis infection by sulphapyridine failed. This experiment showed that attempted combination of both procedures was also valueless.

DISCUSSION.

There has been a tendency in recent years to the replacement of simple dilution methods of virus-titration by methods involving the production of discrete foci on some susceptible tissue. The plaque-counting method of bacteriophage titration was an obvious development from the ordinary methods for making counts of viable bacteria, but in principle it is similar to the analogous methods of animal and plant virus-titration. Plant viruses such as that of tobacco mosaic are now almost invariably titrated by the focal lesions method. The interesting mathematical relationships between concentration of virus and the average counts obtained have been closely studied, e.g. by Samuel and Bald (1933). The method of titration by pock-counts on the chorioallantois was developed in this laboratory (Burnet, 1936), and remains our standard method for those viruses to which it is applicable. With some viruses, however, of which psittacosis is a typical example, the method is unsatisfactory. This virus can be cultivated on the egg-membrane, but the lesions produced are small, irregular in size, and prone to produce secondary foci. We have shown in the present paper that the chorioallantois is also considerably less susceptible to psittacosis virus than are mouse-tissues, either lung or peritoneum.

The data presented in this paper establish the value of intranasal inoculation of mice as an alternative means of focal lesion titration for psittacosis virus. Although our attempts to demonstrate a protective action of chemotherapy or of immunological procedures were negative, the results in themselves provide particularly good indications of the reproducibility of the results.

In several respects psittacosis virus resembles a small bacterium; its elementary particle is considerably larger than any other virus, and its staining reactions are essentially those characteristic of bacteria (Robinow and Bland, 1938). The only other virus resembling it in these respects is that of lymphogranuloma inguinale, and in view of the reported positive results in the chemotherapy of experimental and human infections with that virus (MacCallum and Findlay, 1938) we considered it important to test the activity of members of the sulphanilamide group of drugs against psittacosis. None of the drugs tested (prontosil, sulphapyridine, sulphanilamide, and proseptasine) influenced the lesions produced by intranasal instillation of even minimal amounts of virus.

Previous work on active immunity in mice has given superficially discrepant results. Rivers and Berry (1931) found no evidence of immunity in mice surviving intraperitoneal injection of virus. Fortner and Pfaffenberg, on the other hand, found that survivors would resist a thousand times the dose fatal to controls, and Bedson (1933) definitely increased resistance by immunization with formalin-killed virus. These discrepant findings can, we think, be clarified by consideration of our results in the light of Bedson's paper on immunity to psittacosis in the mouse (1938). He concluded that in mice immunized with dead virus there was no demonstrable resistance to *infection* by virus given intraperitoneally, but that multiplication of the virus stopped short of that required for the production of frank disease. The inapparent infections produced might persist for as long as seven months. We have confirmed Bedson's finding for intraperitoneal test-injections, but find that in equally large groups of immunized mice intranasal inoculations produce as many foci as in controls. It seems therefore that immunity in mice is never sufficient to prevent infection by either route, but may be adequate to reduce the intensity of multiplication below the level necessary to cause death. In such circumstances it is only to be expected that differences in the virulence of the strains used, or in the route of inoculation, might result in what appear to be widely different findings.

The position with neutralizing antibodies in convalescent serum is similar. All workers agree that at most only a small neutralizing effect can be demonstrated. Rivers and Schwentker (1934) found that if relatively large numbers of mice were used, intraperitoneal injections of virus and immune serum gave a significantly increased percentage of survivors and a lengthened survival time in those that died. Bedson (1938) found that virus-serum mixtures which failed to produce symptoms nevertheless gave rise to inapparent persistent infections. Our failure to show any influence of serum by intranasal inoculation accords therefore with previous results.

It may be that this high resistance of psittacosis virus to antibody both *in vitro* and *in vivo* is correlated with the epidemiology of the infection in its natural hosts, wild Australian parrots and cockatoos. All the evidence (Burnet, 1934) suggests that primary infection normally occurs in the nest, being derived from the droppings of infected but healthy parents. For psittacosis virus to persist in nature it is therefore necessary that it should be able to survive in the body of an immune host for at least a year.

A final point of interest is the contrast between the lesions produced by influenza virus and by psittacosis virus in the same situation. There is no production of focal lesions with minimal doses of influenza virus; the small lesions produced correspond fairly obviously to the distribution of a smaller or larger division of the bronchial tree. Presumably influenza virus is strictly limited to the respiratory epithelium, and spread takes place from cell to cell by surface continuity, so that the distribution of alveolar collapse or consolidation is determined by the distribution of the affected bronchioles. In psittacosis the primarily infected cells may be either in the bronchioles or in the alveoli, but there is no limitation of tissue specificity, and inflammatory mononuclear cells in the neighbourhood of the primary lesion become infected. This process continues until a macroscopically visible focus results. The behaviour of psittacosis virus is in this respect more analogous to a low-grade bacterial infection than to the behaviour of more typical tissue-specific viruses.

SUMMARY.

When psittacosis virus is administered intranasally to mice in high dilution, characteristic focal lesions are produced, the number of which can be used as a measure of the concentration of virus.

Using this method of estimating virus activity, it has been shown: (a) that none of the commonly used sulphonamide drugs has any influence on the infection; (b) that mice immunized by several methods failed to show increased resistance to infection by the intranasal route; (c) that no reduction in activity of the virus results when it has been exposed to the action of immune serum *in vitro*.

Immunization with formolized virus, which is ineffective against intranasal infection, produces a definitely increased resistance to intraperitoneal infection.

REFERENCES.

Bedson, S. P. (1933): Brit. J. exp. Path., 14, p. 162.
Bedson, S. P. (1938): *Ibid.*, 19, p. 353.
Burnet, F. M. (1935): J. Hyg., 35, p. 412.
Burnet, F. M. (1936): M.R.C. Spec. Rep. Ser. No. 220.
Fortner, J. and Pfaffenberg, R., cited by Pfaffenberg (1936): Erg. d. Hyg., 18, p. 251.
Hornus, G. (1939): C.R. Acad. des Sciences, 208, p. 675.
Hornus, G. (1940): An. Inst. Pasteur, 64, p. 97.
MacCallum, F. O. and Findlay, G. M. (1938): Lancet, 2, p. 136.
Mauer, G. (1938): Zentralbl. f. Bakt., 142, p. 279.
Rivers, T. M. and Berry, G. P. (1931): J. exp. Med., 54, p. 105.
Rivers, T. M. and Schwentker, F. F. (1934): *Ibid.*, 60, p. 211.
Robinow, C. F. and Bland, J. O. W. (1938): Nature, 142, p. 720.
Samuel, G. and Bald, J. G. (1933): Ann. appl. Biol., 20, p. 70.

INFLUENZA VIRUS INFECTIONS OF THE CHICK EMBRYO BY THE AMNIOTIC ROUTE

2. TITRATIONS AND SERUM NEUTRALIZATION TESTS.

by F. M. BURNET[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Accepted for publication 11th September, 1940.)

Any new method for the demonstration of virus activity, if it is to be of practical use in research or routine work, must be suitable for quantitative estimation both of virus and of the specific neutralizing power of immune sera against the virus in question. This paper is concerned with the potentialities of the amniotic method for titration of influenza viruses and for the demonstration of specific neutralizing antibodies. Of these the first is the more important, as there is otherwise no practical method of titrating virus which has not been adapted (and changed in the process) for growth in mice or on the chorioallantois. Ferrets are too expensive to breed and maintain to allow their use for any extensive quantitative experiments. Neutralization tests, on the other hand, can be carried out very conveniently in mice or eggs with suitably adapted strains of the different antigenic variants. It will be shown that while the amniotic method is very valuable as a method of direct titration it is almost useless for serum-neutralization tests. Actually the results of the duplicate experiments in which the same serum-virus mixtures are inoculated amniotically into chick embryos and intranasally in mice provide very striking evidence of the now well-established fact that antiserum has no directly destructive effect on the virus.

Technique. The inoculation technique has already been described. Titrations were made by the inoculation of serial ten-fold dilutions made in ice-cold saline containing 10 p.c. normal horse-serum. The amounts injected were approximately 0·05 c.c. Owing to the difficulty of calibrating fine-pointed capillary pipettes, it was judged sufficient to estimate the amount by eye, and since we are concerned only with tenfold differences, any error introduced would be insignificant. Eggs were opened at two or more days after inoculation, according to the principles discussed in the preceding paper. A decision as to whether specific infection had occurred was based on (a) death of the embryo at the appropriate period without evidence of bacterial contamination, (b) typical tracheal smear and/or characteristic macroscopic changes in the lungs. Where an end-point was based only on the death of an embryo appropriate subinoculations were made to confirm the result.

Tests for the inactivating power of sera were made according to the methods described by Burnet, Keogh and Lush (1937), the mixtures of appropriately diluted reagents being usually allowed to stand at refrigerator temperature for two hours before inoculation. Tests in mice or on the chorioallantois followed the conventional lines.

1. *Titration of Virus-Containing Material by the Amniotic Method.*

(a) *Unadapted ferret virus.* We have not attempted to determine the infective titres of unadapted virus for ferrets, owing to the prohibitive expense of sufficiently large numbers of animals. As an independent check on the values obtained by amniotic titration, the same dilutions were inoculated intranasally in mice, which were tested a fortnight later for immunity to a mouse-passage strain. The virus used was in the form of amniotic fluid from embryos inoculated with first passage ferret-turbinate emulsion of the strains "Bund." and "R".

Table 1 shows the results obtained by the two methods of titration. The results are quite typical of those obtained with virus strains of this type. There are always a few eggs which die early and from which no definite information can be obtained, and when the infective limit is approached there is an occasional "miss" at dilutions which would be expected to infect with regularity.

[1] Work carried out under grants for research on virus diseases from the National Health and Medical Research Council and from Mr. E. Alec Cato.

TABLE 1.

Comparison of power to infect chick embryos by amniotic inoculation with immunizing power for mice of unadapted influenza virus.

Strain and dilution.		Lesions by amniotic inoculation.	Response of mice to test injection of "Melbourne" virus 1: 100.
"R"	10^{-7}	+, (+), −	
	10^{-6}	+, (+), −, −	44, 44, 46
	10^{-5}	+, +, +, (+)	43, 45, 46, 3
	10^{-4}		47, 0
	10^{-3}		2, 1, 0, 0
	10^{-2}		0
	10^{-1}		0, 0
"Bund"	10^{-7}	−	
	10^{-6}	+, +, −	44, 46, 45, 45
	10^{-5}	+, +, +, +	44, 44, 45, 46
	10^{-4}		44, 44, 46, 3
	10^{-3}		45, 47, 2, 2
	10^{-2}		44, 2, 1
	10^{-1}		47, 1, 1, 0

+ = definite infection with typical tracheal fluid smear.
(+) = embryo dying on third or fourth day, probably from specific infection.
− = embryo surviving to fourth or fifth day without evidence of infection.
Early deaths or embryos showing bacterial contamination are not recorded.
Mice lesions are recorded according to the usual convention.
Those which indicate partial or complete immunity are in heavy type.

Both fluids are infective to a dilution beyond 1: 10^6 by the amniotic method. Neither produced typical lesions in mice, although there was a number of deaths amongst mice given the lower dilutions of strain "R". *Post mortem*, these animals show at most only small areas of consolidation in the lungs, and the deaths were considered to be of nonspecific origin. Tests with undiluted material in mice gave a proportion of one- and two-grade lesions when the lungs were examined at seven days. The unadapted strain "Bundoora" gave even less, only doubtful one-grade lesions being found. Despite the absence of lesions, infection of the mouse lung evidently occurs with both strains, as judged by the development of immunity. At least 1,000 minimal infective doses by the amniotic route are necessary to induce immunity. As far as can be judged from the rather incomplete data, it requires a larger amount of "Bundoora" virus than "R" virus to immunize, and it is clear that the grade of immunity produced is lower.

This experiment is of interest from several points of view. In the first place it shows that unadapted virus can infect mice fairly readily, although no lesions are produced. This may provide a simple means not hitherto used to detect influenza virus in human throat washings, viz. by inoculation of filtrates intranasally in mice with a test for immunity fourteen days later with a standard strain. Second, it provides an example of a quantitative experiment quite impracticable by any method other than the amniotic technique, and finally, it indicates the very high virus titre obtained in the fluids of embryos inoculated by this route. We have never encountered a fluid either amniotic or allantoic which was not infective at a dilution of 10^{-6}, and have frequently obtained infection at a dilution of 10^{-8}.

(b) *Mouse virus.* All the mouse-passage strains we have tested will infect amniotically, and in Table 2 is shown the result of a comparative titration of "Melbourne" strain by the amniotic method and in mice. The virus was prepared in the usual way by grinding one mouse lung removed two days after inoculation, with quartz powder and 5 c.c. of broth. Dilutions were made from the supernatant fluid after centrifugation.

TABLE 2.

Comparative titration of "Melbourne" mouse strain amniotically and by intranasal inoculation in mice.

	Virus dilutions.					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Amniotic method			+, +, (+)	(+), −	+, +	+, +
Mice intranasally	44, 44, 46	44, 46, 46	46, 47, 47	3, 3, 2	2, 2, 1	

This preparation was rather more active than was expected, and the end-point was not reached with either method.

The results are sufficient to show that the amniotic method is at least as sensitive as the mouse intranasal technique, even with a strain long adapted to and highly virulent for mice.

(c) *Egg passage virus.* Our standard egg passage strain "Melbourne" differs from all the other influenza strains tested in failing to give infections of the respiratory tract. When inoculated amniotically, multiplication takes place probably in the cells of the amnion and rapid blood-borne infection of the embryo follows, with death in two, or at most three days. Comparative titrations by amniotic and chorioallantoic routes gave approximately the same end-points. With this strain there is no advantage of the amniotic method over the standard one.

The other well-adapted egg strain "W.S." (egg) however, behaves normally, producing typical lesions of the respiratory tract, except in an occasional embryo dying early which shows only the septicaemic type of lesion. The end-point of titrations by amniotic and chorioallantoic methods is similar.

2. *Serum-Neutralization Experiments by the Amniotic Technique.*

Two consistent findings of considerable interest have emerged from attempts to carry out neutralization experiments with immune serum by the amniotic method: first, even with mixtures which by other tests are greatly over-neutralized infection still occurs in a proportion of embryos; and second, that with a well-adapted amniotic passage strain, immune serum, although it does not prevent infection, protects the embryo against the normally lethal action of the virus.

A typical experiment with the amniotic passage strain 'Bur.' and homologous ferret immune serum is shown in Table 3.

TABLE 3.

Failure of neutralization by immune serum in amniotically inoculated embryos.

Virus + serum mixtures.	Amniotic test.	Mouse intranasal test.
"Bur." + F.122 1: 2	+, +, +	0, 0, 0
F.122 1: 10	(+), (+), (+)	0, 0, 0
F.122 1: 100	+, +, (+)	3, 3, 3
Saline	(+), (+), (+)	44, 45, 46

The same virus strain and serum were retested using in addition mixtures of ferret serum with dilute virus. Table 4 shows the results.

TABLE 4.

Action of serum on diluted virus of amniotic route.

Virus + serum mixtures.	Amniotic test.	Mouse intranasal test.
"Bur." 10^{-3} + F.122 1: 2	+, −, −	
10^{-2} „ 1: 2	−, −	
10^{-1} „ 1: 2	+, −	
Undiluted „ 1: 2	+, +	0, 0, 0
„ „ 1: 10		0, 0, 0
„ „ 1: 50		3, 2, 1
"Bur." 10^{-4} alone	+, +	1, 0, 0
10^{-3} „	(+), (+)	2, 1, 0
10^{-2} „	+, +	45, 2, 2

This is characteristic of experiments in which a large excess of immune serum is present in the mixtures. There is a significant proportion of embryos which fail to become infected, but no regularity in the results.

Even when the time of contact between serum and virus is prolonged to two days before the inoculations are made there is very little change in the degree of inactivation. Table 5 presents another experiment with the same reagents in which mixtures were tested at two hours and again two days after.

It will be seen that there is no significant change either in the amniotic or the mouse results when the time of contact between serum and virus is increased to forty-eight hours.

TABLE 5.

Attempted inactivation by long contact between serum and virus.

Virus-serum mixture.	Amniotic test. 2 hours.	Amniotic test. 2 days.	Mouse intranasal test. 2 hours.	Mouse intranasal test. 2 days.
"Bur." + S.122 1: 2	+, +, (+)	+, +, —	0, 0, 0	0, 0, 0
S.122 1: 10		+, +, —	1, 0, 0	1, 1, 0
S.122 1: 50		+, +, (+)	2, 2, 1	1, 1, 0
"Bur." 10^{-3} alone	+, +, +	+, +, +	1, 1, 1	1, 1, 1
10^{-2} „	+, (+), (+)	+, +, +	2, 2	2, 2, 2

Although infection is not prevented by immune serum, there is a significant prolongation of the time for which the infected embryos survive. In the experiment shown in Table 6 all the eggs were incubated after inoculation for six days or until death of the embryo occurred. Embryos dead at twenty-four hours are not noted in the table. All the other deaths were apparently specific. Parallel tests with the same mixtures were made in mice, but it should be recognized that the strain used, fortieth amniotic passage "R", had never previously been in mouse tissues, and was of only moderate and rather irregular virulence for mice.

TABLE 6.

Action of immune serum in allowing survival of infected embryos.

Virus-serum mixture.	Amniotic.	Mouse intranasal.
"R" 1: 100 + S.129	†4, †5, +6	0, 0, 0
1: 10 + S.129	†3, †4, †5, †5	0, 0, 0
1: 10 + S.129 1: 5	†4, †4, †5	0, 0, 0
1: 10 + S.129 1: 25	†2, †2, †2, †2, †2	2, 1, 1
1: 10 + saline	†2, †2, †2, †4	47, 3, 1
"R" 10^{-6}	†3, †4, —5	
10^{-7}	†3, †3	
10^{-6}	†2, †3, †3	0, 0, 0
10^{-5}	†3, †3	3, 1, 0
10^{-4}		2, 1
10^{-3}		3, 3, 0

† = specific death of embryo.
+ = specific virus lesions in surviving embryo on day shown.
— = no lesions in embryo examined on day shown.

To complete this experiment the same serum and similar virus, forty-third amniotic passage "R", were tested, using virus diluted serially from 1: 100. The survivors were examined five days after inoculation.

TABLE 7.

Action of immune serum on low concentrations of virus.

Virus-serum mixture.	Amniotic test.
"R" 1: 10^5 + S.129	†4, —5, —5, —5
1: 10^4 + S.129	+5, —5, —5, —5
1: 10^3 + S.129	+5, —5
1: 10^2 + S.129	†4, †5, +5
1: 10^6 + saline	†3, †3

Symbols as in Table 6.

In this experiment we have one embryo certainly infected with a mixture of virus 1: 10^4 and undiluted serum, while in mice 1,000 times the amount of virus is neutralized by serum diluted 1: 5.

All the experiments described so far were made with amniotic passage virus, since our main preoccupation was with the hypothetical problem: if an unknown respiratory virus were isolated by the amniotic method, how could serological studies be carried out? It was of interest to apply the method to influenza virus strains which had been maintained by mouse passage, and to use serum-mouse virus mixtures. With such material a much closer correspondence between amniotic and mouse lung tests was evident, but in practically every experiment an occasional embryo was infected with mixtures which were completely inactive for mice. This difference is not due to the nature of the material in which the virus is suspended, but to a functional change in the character of the virus itself. Table 8 shows the result of experiments in which the mouse

strain "R" was tested as mouse virus ("R.M.V.") and in the form of amniotic fluid from embryos inoculated with mouse virus (1st "R.M.A.P.") against an homologous ferret serum, "F.97".

TABLE 8.

Comparison of mouse virus with first amniotic passage material.

Serum-virus mixtures.	Amniotic test.	Mouse intranasal test.
"R" M.V. + F.97 1: 2	+, −, −	0, 0, 0
1: 10	(+), −, −	0, 0, 0
1: 50	+, +	2, 1, 1
"R" M.V. 1: 100	+, +	47, 3, 3
1: 1,000	+, (+)	2, 2, 2
1: 10,000	+, +	2, 1
"R" M.A.P. + F.97 1: 2	−, −	0, 0, 0
1: 10	−, −, −	0, 0, 0
1: 50	−, +	0, 0, 0
"R" M.A.P. 1: 100	+, +	47, 3, 2

This result should be contrasted with the behaviour of "R" amniotic passage strain with a similar though slightly weaker homologous immune serum shown in the previous two Tables.

"Melbourne" virus fully adapted to the chorioallantois appeared to be fully neutralized when tested by the amniotic method. When a mixture of virus (titre 2×10^5) with undiluted homologous serum was tested on the chorioallantois an average of seven foci per membrane was obtained. By the amniotic route the same mixture gave one specific death and two survivals. No specific deaths were obtained with virus 1: 10 and 1: 100 plus immune serum.

DISCUSSION.

An extensive experience of several thousand amniotic inoculations is sufficient to allow the statement that once the technique has been mastered titrations are as convenient and satisfactory as any other method of animal inoculation which gives a simple positive or negative result. We are at present applying the method to determining the amount of virus which can be washed from the nasal passages of ferrets at different stages of infection. There seems to be no reason why it should not be equally applicable to similar studies in human beings and so supply data not at present available which might provide an important help toward understanding the epidemiology of influenza.

The serological studies reported are of considerable theoretical interest. It has been established for some years that the interaction of a typical virus with homologous immune serum does not result in irreversible destruction of the virus. The apparent inactivation is a result in which the virus, the specific antibody, and the susceptible cells of the test organism are all concerned. Discussions of the general problem with references will be found in the monograph by Burnet, Keogh and Lush (1937) and in the article by Hallauer (1939). One of the most decisive ways of demonstrating that a serum-virus mixture, non-infective for some standard test animal, still contains active virus is to produce infection with the same mixture by some other route of inoculation or in some other organism. The example shown in Table 7, in which infection of the chick embryo was produced by a mixture with a serum : virus ratio 5,000 times that in a mixture completely non-infective for mice is probably the most striking one yet published.

Such a result was obtained using virus adapted to multiply by the amniotic route by more than 40 consecutive amniotic passages. Mouse virus of the same original strain was much more readily inactivated by immune serum, the results by the amniotic route being not greatly discrepant from those in mice. Evidently a change has occurred in the virus by virtue of which infection of cells of the respiratory tract by virus partially coated with antibody globulin is greatly facilitated. Discussion of such changes in character on passage will be deferred to a later paper dealing specifically with the changes in influenza virus activity associated with continued amniotic passage.

Although immune serum has very little effect in preventing infection by amniotic passage strains, it allows a considerable prolongation of the life of the embryo. In most respects the fate of an embryo infected with passage virus plus serum is the same as that of an embryo infected with dilute unadapted virus alone. We have at present no indication as to why amniotic infections with passage strains kill the embryo so rapidly, and very little can therefore be said about this effect of serum. There are certain analogies to be found in the behaviour of rickettsiae and psittacosis virus with homologous immune serum. In both cases symptoms may be reduced or a longer period of survival result without any evidence that infectivity is destroyed.

SUMMARY.

The amniotic method may be used for the titration of any type of influenza virus.

Unadapted virus administered intranasally to mice may provoke immunity without producing visible lesions.

With mouse and chorioallantoic passage strains the titre determined by amniotic titration is approximately the same as that obtained by standard methods.

With strains adapted to amniotic passage there is very little effect of contact with immune serum apart from a prolongation of the survival period of the embryo.

With unadapted strains inactivation by serum can be demonstrated using amniotic inoculations.

REFERENCES.

Burnet, F. M., Keogh, E. V. and Lush, D. (1937): Austral. J. exp. Biol., 15, p. 227 (Supplement).
Hallauer, C. (1939): In Doerr and Hallauer *Handbuch der Virusforschung*, Wien, p. 1147.

A STUDY OF SOME AUSTRALIAN STRAINS OF *ERYSIPELOTHRIX*

by NANCY ATKINSON

(From the Institute of Medical and Veterinary Science, Adelaide).

(Accepted for publication 20th November, 1940.)

In a previous paper, Atkinson and Collins (1940) reported swine erysipelas in South Australia. Various strains of *Erysipelothrix* were submitted to detailed cultural and serological investigation. The cultural reactions corresponded well with those described by other workers, but antigenically the strains of *Erysipelothrix* did not form a homogeneous group. As many Australian strains as possible were collected, therefore, from this and other States[1], for detailed examination. The results demonstrated the existence of at least two antigenically-distinct groups.

Of 33 Australian strains, 13 (E2, E3, E5, E8, E9, E11, E13, E16, E17, E37, E39, E40, E43) were isolated during 1939 and 1940 in South Australia from pigs suffering from swine-erysipelas, in chronic, urticarial or septicaemic form. E44 was obtained from a pig in Victoria, E19–E37 from New South Wales. Strains E19–E28 were isolated from cases of ovine arthritis between 1924 and 1928, E29 from ovine arthritis in 1939. E30–37 were pig-strains of which E31–E34 were isolated from cases of porcine arthritis during 1939. One English strain, No. 1224 N.C.T.C., was labelled E1.

Cultural reactions. Only smooth forms of *Erysipelothrix* were investigated.

After growth in serum-broth for 18 to 24 hours, followed by plating on horse-blood-agar, a typical smooth colony was cultivated in serum-broth. In this medium rough variants rarely arose, even after two months. Stock-cultures were preserved therefore in serum-broth. In partly rough cultures, the rough variant was usually predominant at or above 37° C., while at 30°–32° C. the growth of the smooth variant was favoured. Therefore, all cultures were incubated at 30°–32° C.

Cultures in serum-broth and on blood-agar consisted of small, slender, Gram-positive rods arranged singly or in short chains and often showing irregular staining. All were non-motile.

In serum-broth a uniform suspension appeared in 24 hours and gradually settled out; the sediment could readily be resuspended on shaking. In ordinary broth in 24 hours the turbidity was less. On blood-agar in 24 hours all strains showed typical smooth colonies, water-clear and circular with glistening surface, entire margin and surrounding zone of greenish discolouration. Growth on ordinary agar was poor and the colonies were often rough. On MacConkey's agar there was no growth. In a gelatine-stab growth occurred slowly at 18° C. and lateral outgrowths of transparent fluffy appearance formed after 1–4 weeks. Acid was produced from glucose, maltose and lactose, but not from sucrose or mannitol.

Cultures dried over P_2O_5 *in vacuo* remained viable for at least three months and yielded only smooth organisms.

Serological reactions. The 34 strains of *Erysipelothrix* were submitted to agglutination and agglutinin-absorption tests, using antisera prepared against several of these strains.

Antisera. Heat-killed (56° C for 30 min.) suspensions of washed organisms were prepared in saline from 24-hour cultures in serum-broth and standardized to an opacity corresponding to approximately 1300×10^6 *Bact. coli* per ml. Graded amounts (0·1 ml.–1 ml.) were injected intravenously into rabbits on eight successive days. The animals were bled on several occasions between the ninth and sixteenth days after the first injection, and the sera preserved with 0·25 p.c. phenol.

Suspensions. These consisted of 24-hour broth-cultures containing 0·25 p.c. formalin and killed at 56° C. in 30 minutes. In a large series of tests, this type of suspension gave results comparable with or slightly better than live broth-cultures and did not show auto-agglutination. Saline-suspensions of organisms from cultures in serum-broth showed some precipitation.

The suspensions corresponded in opacity to that of approximately 350×10^6 *Bact. coli* per ml.; they remained stable for at least two to three months. All new suspensions were standardized against the old one, and generally possessed the same agglutinability; if not, they were discarded.

---

[1] Many workers have assisted me in obtaining these strains.

Technique of agglutination tests. To serial two-fold dilutions of antiserum in saline an equal volume of suspension was added and the mixture placed in a water bath at 55° C. Agglutination, which was of the granular O type, was complete after 18 hours. Complete agglutination was taken as the end point.

Technique of agglutinin-absorption tests. Serum in a concentration 200 times its titre was mixed thoroughly with an equal volume of saline-suspension of washed organisms from a serum-broth culture and allowed to stand overnight at 2° C. After centrifuging, the clear supernatant liquid was used for agglutination tests. Where necessary, this supernatant liquid was absorbed a second time. Controls were included.

Results of agglutination tests. The first set of tests performed with four different antisera and the homologous organisms, E1, E2, E3 and E5, are shown in Table 1. Antisera E2, E3 and

TABLE 1.

Results of agglutination tests.

	Reciprocal titre with antiserum prepared against			
Antigen.	E1.	E2.	E3.	E5.
E1	2,560	320	640	20,480
E2	—	2,560	1,280	2,560
E3	—	1,280	1,280	1,280
E5	1,280	640	640	20,480

— = no agglutination at a dilution of 1/20 or more.

E5 agglutinated all four strains to varying titres; antiserum E1 failed to agglutinate E2 or E3. Thus the strains could be divided into two groups by agglutination tests with E1 serum, E1 and E5 forming one group and E2 and E3 the other. A similar division is indicated less clearly by the other sera.

All strains of *Erysipelothrix* were then tested against E1, E2 and E5 sera selected as representative of three possible types. Table 2 indicates the existence of three serological groups.

TABLE 2.

Results of agglutination tests.

	Reciprocal titre with antiserum prepared against		
Antigen.	E1.	E2.	E5.
E8	2,560	640	10,240
E13	—	1,280	2,560
E16	320	1,280	2,560
E21	1,280	640	10,240
E34	160	1,280	2,560
E40	—	1,280	2,560
E44	2,560	640	20,480

— = no agglutination at a dilution of 1/20 or more.

TABLE 3.

Results of mirror absorption tests.

			Reciprocal titre after absorption with			
Antigen.	Serum.	Titre unabsorbed.	E1.	E2.	E3.	E5.
E1	E1	2,560		2,560	2,560	—
E2	E1	—		—		
E3	E1	—		—	—	
E5	E1	1,280		1,280		—
E1	E2	320	—			
E2	E2	2,560	1,280		—	160
E3	E2	1,280	1,280		—	
E5	E2	640	320			—
E1	E3	640	—			—
E2	E3	1,280		—		320
E3	E3	1,280	1,280	—		640
E5	E3	640				—
E1	E5	20,480	—	20,480		
E2	E5	2,560	640	—		
E3	E5	1,280	320	—	—	
E5	E5	20,480	640	2,560	2,560	

— = no agglutination in any dilution tested; the lowest dilution was 50 × titre.

The first E1 group (including E8, E21 and E44) was agglutinated by E1 and E5 sera to full or half-titre and by E2 serum to less than half-titre. The second or E2 group (including E13 and E40) was agglutinated to full or half-titre by E2 serum and to one-eighth titre or less by E5 serum, but not by E1 serum. These two groups correspond with those already indicated by the previous tests. The third group (including E16 and E34) resembled the first in that it was agglutinated to some extent by all three sera, but agglutination to full or half-titre occurred only with E2 serum; the other two sera, E1 and E5, produced agglutination to one-eighth titre or less. This group, therefore, is most closely related to group 2.

There were 13 strains in group 1, 8 in group 2, and 13 in group 3.

Results of agglutinin-absorption tests. The four strains, E1, E2, E3 and E5 and their homologous antisera were used in a set of "mirror tests" in which each serum was tested by absorption with each strain except the homologous one. (Table 3.)

The "mirror test" with E2 and E3 showed that these two organisms contained the same antigenic components; agglutinins for the homologous organism were removed completely by absorption with the other.

With E1 and E2 or E1 and E3 the cross-absorption tests revealed little antigenic relationship; absorption with the heterologous organism either had no effect on the titre for the homologous organism or caused only slight reduction. In these tests E2 and E3 again behaved similarly, thus confirming the suggestion that they were antigenically identical.

The antigenic distinction between E5 and the other strains was not so clear-cut. Cross-absorption tests with E1 showed a marked sharing of antigens. E5 completely removed agglutinins for the homologous organism from E1 serum, indicating that E5 contained all the antigens of E1; the two organisms were not antigenically identical, however, because absorption of E5 serum with E1 did not completely remove agglutinins for E5 although their titre was very markedly reduced. Thus E5 contained a major antigenic component similar to that characteristic of E1, and also another antigen not present in E1. Absorption tests with E2 and E3 showed the presence in E5 of the other antigenic component found in E2 and E3; absorption of E5 serum with either E2 or E3 caused a marked reduction in titre for E5 and absorption of E2 or E3 serum with E5 also caused a reduction in titre. Thus E5 is related antigenically to E1, E2 and E3.

The results of these absorption tests again indicate two antigenic types of *Erysipelothrix* represented by E1 and E2 respectively, and an intermediate type, E5, related to both.

Results of agglutination tests with absorbed sera. E1 serum was absorbed with E2 and E2 serum with E1; these sera then agglutinated only the homologous organisms, and were therefore specific. All strains of *Erysipelothrix* were tested by agglutination with these specific sera. Typical results are given in Table 4.

TABLE 4.

Results of agglutination tests with absorbed sera.

	Reciprocal titre with Strain											
Serum.	E1.	E2.	E3.	E5.	E8.	E13.	E16.	E21.	E31.	E34.	E40.	E44.
E1 absorbed	2,560	—	—	640	640	—	—	1,280	1,280	—	—	1,280
E2 absorbed	—	640	640	160	160	640	640	640	—	320	640	40

— = no agglutination at a dilution of 1/40 or more.

With the specific sera the three groups indicated by the mirror tests appeared; firstly the E1 group (E1 and E31) agglutinated by E1 serum but not by E2 serum, secondly the E2 group agglutinated by E2 serum but not by E1 serum (E2, E3, E13, E16, E34 and E40), thirdly the E5 group agglutinated by both E1 and E2 sera (E5, E8, E21 and E44). E26 was not agglutinated by either serum and could not be typed.

To test further the presence of both E1 and E2 type antigens in E5, absorption tests were made with E5 serum. This serum after complete absorption with E1 still agglutinated E2 and E5, and after complete absorption with E2 still agglutinated E1 and E5 (see Table 3).

Strains of the E1 group were rare; only one, E31, was found among the Australian *Erysipelothrix*. E1 itself, as already noted, was an English strain. There were 20 strains in the E2 group. The E5 group contained 11 strains.

These results confirm the presence in the *Erysipelothrix* group of two different antigenic factors represented in E1 and E2 respectively and occurring together in the E5 type of *Erysipelothrix*.

Tests with heated antigens. Watts (1940) obtained two distinct serological groups of *Erysipelothrix* by direct agglutination with antisera prepared against and tested with suspensions steamed for four hours. Heated suspensions of E1 and E16 were prepared by Watts' method, using 1/2000 merthiolate as antiseptic. For comparison, suspension containing 0·25 p.c. formalin was also steamed. As controls, unheated merthiolate- and formalin-suspensions, and a fresh saline-suspension of living organisms were included in tests performed with the E1 and E2 sera used in all the previous experiments. To obviate possible differences in agglutinability from one subculture to the next all suspensions were prepared with organisms from a single serum-broth

culture of the required strain. The preparation of sera against heated organisms was not considered necessary, as serum prepared in the standard way should contain antibodies for all the important antigenic factors, heat-stable and heat-labile, in the organism. The results of these tests are given in Table 5.

TABLE 5.

Results of agglutination tests with various antigens.

Antigen.	Reciprocal titre with antiserum prepared against E1.	E2.
E1 merthiolate heated	640	40
E1 formalin heated	2,560	160
E1 merthiolate unheated	1,280	160
E1 formalin unheated	2,560	640
E1 saline unheated	2,560	640
E16 merthiolate heated	20	320
E16 formalin heated	40	1,280
E16 merthiolate unheated	80	640
E16 formalin unheated	160	1,280
E16 saline unheated	160	1,280

The strains E1 and E16 were selected for this test because they represented the antigenic types E1 and E2 respectively, but both showed cross-agglutination with unabsorbed sera (see Tables 2 and 4). This cross-agglutination still occurred with the heated suspensions which, however, gave a lower titre than the unheated ones (Table 5). Both heated and unheated suspensions containing formalin gave higher titres than those containing merthiolate; unheated formalin-suspension gave the same results as the fresh saline-suspension.

Extraction of the specific antigen. Extraction of the organisms with hot dilute acetic acid produced a solution which gave a positive ring-test with homologous antiserum.

A suspension of the organisms from 100 ml. of a 24-hour serum-broth culture in 5 ml. of N/30 acetic acid was placed in a boiling water bath for 45 minutes, cooled, neutralized with NaOH and centrifuged. For the ring-tests 0·3 ml. of this clear extract was layered in a Dreyer tube on 0·3 ml. of antiserum diluted 1/3 in saline. Readings were made hourly for five hours with mixtures at room temperature (about 18° C.). A final reading was made after the tubes had been kept at 2° C. overnight. After five hours at 18° C. the results, some of which are given in Table 6, were easily read and showed a marked specificity of reaction.

TABLE 6.

Results of ring tests with acetic acid extracts.

Antiserum prepared against	Extract of Strain E1.	E2.	E5.	E26.	E31.	E39.	E40.	E44.
E1	+	—	+	+	+	+	—	+
E2	—	+	—	—	—	—	+	—
E5	+	—	+	+	+	+	—	+

+ = ring present after 5 hours at 18° C.

The extracts formed two groups. One corresponding to the E1 type of *Erysipelothrix* gave a positive test with E1 and E5 serum but not with E2 serum; the other representing the E2 type of *Erysipelothrix* gave a positive test with E2 serum but not with E1 or E5 serum. The intermediate or E5 type of *Erysipelothrix* was not detected by the ring-tests at this time and organisms of this group fell into the E1 group. However, after standing overnight at 2° C., a weak reaction was given by the E5 extract with E2 serum. Similar results were found with the extract of E21 (an E5 type by agglutination), but E44 (also an E5 type by agglutination) gave no reaction with E2 serum. Table 4 shows that although both E1 and E2 type-sera agglutinated E44, the titre was low with the E2 serum, suggesting that only a small amount of E2 type antigen was present. This was not detected in the extract.

All extracts gave a strongly positive Molisch test, and the active substance could be precipitated by the addition of two volumes of alcohol. The specific substance appears to be a carbohydrate.

Tests with extracted organisms. Satisfactory suspensions of organisms extracted with acetic acid could not be made. All showed some degree of auto-agglutination in saline and many even in distilled water. Agglutination tests with E1, E2 and E5 sera were performed with all suspensions which appeared relatively stable. No agglutination occurred except where the control was auto-agglutinated.

PATHOGENICITY TESTS.

In mice the strains tested produced characteristic conjunctivitis and the animals died 2–4 days after inoculation with an amount as small as 0·001 ml. of broth culture. Organisms of E2 type killed mice most rapidly.

Inoculated intravenously and intradermally into a pig in a large dose, E2 produced an acute illness described fully elsewhere (Atkinson and Collins, 1940). The animal appeared to recover completely, increasing rapidly in size and weight, but eight weeks after inoculation it suddenly died. Post-mortem examination revealed on the heart-valves large vegetations characteristic of chronic swine erysipelas. *Erysipelothrix* of E2 type was isolated in both rough and smooth forms from these lesions and from other organs. E2 type of *Erysipelothrix* is pathogenic therefore for pigs and causes typical chronic swine erysipelas. Intradermal inoculation of E2 produced within a few hours a pronounced local reaction. Tests in the pig were made therefore with strains of *Erysipelothrix* representative of the various serological types to determine if any particular type was associated with this reaction. The sediment from 5 ml. of a 24-hour serum-broth culture was suspended in 0·5 ml. of saline and 0·2 ml. was injected intradermally. A positive reaction appeared in a few hours as a reddish-purple raised area several centimetres across. All the strains tested gave positive results.

None of the strains tested was pathogenic for guinea-pigs.

DISCUSSION.

The morphological and cultural characteristics of Australian *Erysipelothrix* agree well with those recorded for other strains. There has been some divergence in the biochemical reactions obtained by various workers. All our cultures fermented glucose, lactose and maltose. Deem and Williams (1936), using a special medium, did not obtain fermentation of maltose with any of their 37 strains, nor did Barber (1939) with six strains. On the other hand, Watts (1940) working with 43 strains of varied origin recorded acid production from maltose by all.

Topley and Wilson (1936) mention that *Erysipelothrix* is very susceptible to desiccation, but the cultures tested here remained viable for at least three months after drying *in vacuo*.

Serological tests demonstrated that the *Erysipelothrix* group was not antigenically homogeneous (see Topley and Wilson, 1936), but contained two different antigens specific for types E1 and E2 respectively. Strains could be typed by the use of specific absorbed sera, when an intermediate antigenic type (E5) containing both specific antigens, with that of E1 predominating, was discovered. The distinct types E1 and E2 may correspond to the groups described by Watts (1940), who did not record intermediate types. The cross-agglutination which occurred with unabsorbed serum may have been due to the presence of a group-antigen distinct from the specific antigens and shared by all types, or to the presence in a specific organism of a small amount of heterologous type-antigen resulting in the production, during immunization, of both E1 and E2 antibodies. The latter explanation seems the more probable. There is a strong resemblance between the antigenic picture of the *Brucella* (see Wilson and Miles, 1932) and that of the *Erysipelothrix*.

The E2 type of *Erysipelothrix* was the most virulent for mice and caused chronic swine erysipelas in a pig; pathogenicity, however, was not associated particularly with any antigenic type, as both E1 and E5 types killed mice though more slowly than E2. Ovine and porcine strains of *Erysipelothrix* could not be differentiated on antigenic type as they included both E2 and E5 types. The E1 type was found only once among the Australian strains; this strain was E31 from a case of porcine arthritis.

Apart from the rarity of E1 types, there appears to be no correlation between antigenic type and the form of natural infection in the pig; both the E2 and E5 types have been isolated from cases of septicaemic and urticarial swine erysipelas and of arthritis. In the diagnosis of *Erysipelothrix* infections by the agglutination test with the animal's serum one should include in the test suspensions representative of the various types of *Erysipelothrix*.

SUMMARY.

Thirty-three Australian strains of *Erysipelothrix* were investigated. Culturally and morphologically these strains resembled those described elsewhere. Serological tests showed the presence of two distinct antigenic types of *Erysipelothrix* and a third intermediate type. Strains could be typed by agglutination with specific absorbed sera. A specific substance, probably a carbohydrate, was extracted from the *Erysipelothrix* of each type.

The results of pathogenicity tests showed no particular relationship between antigenic type and virulence. The antigenic type could not be correlated with the source of the culture.

REFERENCES.

Atkinson, N. and Collins, F. V. (1940): Austral. vet. J., 16, p. 193.
Barber, M. (1939): J. Path. Bact., 48, p. 11.
Deem, A. W. and Williams, C. L. (1936): J. Bact., 32, p. 303.
Topley, W. W. C. and Wilson, G. S. (1936): The Principles of Bacteriology and Immunity, London, Edward Arnold & Co.
Watts, P. S. (1940): J. Path. Bact., 50, p. 323.
Wilson, G. S. and Miles, A. A. (1932): Brit. J. exp. Path., 13, p. 1.

STREPTOCOCCUS PYOGENES (GROUP A): A REVIEW OF 1,341 STRAINS COLLECTED FROM VARIOUS HUMAN SOURCES

by E. V. KEOGH, R. T. SIMMONS AND HAROLD WILSON

(From the Commonwealth Serum Laboratories, Melbourne).

(Accepted for publication 6th December, 1940.)

During the past three years 1,341 strains of *Streptococcus pyogenes* (Group A) have been collected in this laboratory. Progress reports of the work have been published (Keogh and Kelsey, 1939; Keogh, Macdonald, Battle and Puckey, 1939; Keogh, Macdonald, Battle, Simmons and Williams, 1939(a); *idem*, 1939(b); Macdonald, Simmons and Keogh, 1940; Keogh and Simmons, 1940).

The purpose of this paper is to discuss some technical methods used, to give the results of further work and to collect scattered facts about the local serological types of Australia.

Remarks on technique. After trying several technical methods (Griffith, 1934; Pauli and Coburn, 1937; Ward and Rudd, 1938; Rudd, White and Ward, 1939) for the isolation and serological typing of streptococci, those of Ward and his collaborators were adopted.

(a) β haemolytic streptococci were more easily recognized on the blood-agar plates containing neo-peptone, horse serum and horse blood, recommended by these authors, than on the usual horse blood-agar plates containing meat infusion. Also, this medium with the addition of maltose in place of horse serum, was useful and reliable as a means of distinguishing streptococci of group A from group B and from group C or G of human origin. (Simmons and Wilson, 1941.)

(b) Agglutinating sera were prepared by the method of Rudd, White and Ward (1939) except that the formalinized deposit from 5, 10 and 15 ml. volumes of broth culture was injected in consecutive weeks. Satisfactory titres were usually attained after three injections.

(c) Satisfactory suspensions for slide agglutination were generally obtained by cultivation at 30° C. in the tryptic digest broth of Pauli and Coburn (1937) as modified by Ward and Rudd (1938). Unsatisfactory suspensions were usually made workable by tryptic digestion with the pancreatic extract of Cole and Onslow, as recommended by V. D. Allison (unpublished) and by Ward and Rudd (1938) or with a solution of trypsin, 1 p.c. in distilled water. We believe that, after tryptic digestion, false agglutination seldom occurs with agglutinating sera adequately absorbed with heterologous strains.

(d) The reactions of the unknown strain in serum-broth and serum-peptone-agar and in starch, mannitol and cellobiose indicated the agglutinative type (Table 4). The result of agglutination was confirmed by physiological tests. As previously reported, colonial and biochemical reactions assisted differentiation between closely-related serological types.

(e) Sherman (1937) showed that aesculin broth was of possible differential value with group A strains. Following the successful use of a modified aesculin broth in the classification of group C strains of human origin (Simmons and Keogh, 1940) aesculin broth was used as a further differential medium for group A strains. We confirmed the results of Sherman, but were unable to develop a medium which gave consistent results within 24 or 48 hours. Most strains split aesculin slowly.

(f) Fuller and Maxted (1939a, 1939b) believed that type 3 differed from other types in not forming hydrogen peroxide; by colony selection, however, Hadley and Hadley (1940) obtained variants which formed hydrogen peroxide. Our only type 3 strain, "Lewis", sent to us by Dr. Griffith, behaved similarly. All other types except "Stewart", of which 30 strains were examined, produced peroxide on benzidine blood-agar or chocolate agar.

Distribution of types isolated from various sources. Table 1 shows the sources and type-distribution of the 1,341 strains collected during 1937–40. None of the nine types of Griffith (1934) mentioned at the foot of the table has been met. Of the 13 new ones, four, types "Wade", "Gerrard", "Hooper" and "Krone", were established in Sydney by Rudd, White and Ward (1939); "Hooper" and "Krone" types have not been isolated by us in Melbourne; the remaining nine were recognized in this laboratory.

Certain types were associated predominantly with certain diseases, whereas other types were associated indifferently with various diseases.

In Melbourne, types 2, 17, "Wade" and 11 were the predominant scarlatinal types, whereas 6, "Stewart", "Hempston", 27 and "Alli" were the predominant types in both

TABLE 1.

The numbers and sources of the strains of Griffith's and local serological types collected in Melbourne, 1937–40.

	1	2	3	4	5	6	7	8	9	
Type.	Scarlet fever. Melbourne.	Scarlet fever. Adelaide.	Excised tonsils. Melbourne.	Excised tonsils. Adelaide.	Puerperal and post abortive. Melbourne.	Throat swabs, normal subjects. Melbourne.	Orphanage scarlet fever, contacts and normal subjects. Melbourne.	Surgical. Melbourne.	Surgical. New Zealand.*	Total.
1	—	1	3	7	4	1	—	4	11	31
2	103	1	11	—	—	—	85	10	—	210
4	4	2	11	18	1	1	3	1	1	42
5	—	—	—	—	2	—	—	—	—	2
6	6	1	38	6	1	12	21	38	—	123
8	—	—	—	2	—	—	—	—	3	5
9	—	—	1	10	1	1	—	7	—	20
10	2	1	—	9	—	—	—	1	—	13
11	20	5	6	9	3	1	4	13	2	63
12	—	1	5	7	2	—	—	12	6	33
17	78	3	6	2	1	1	—	11	—	102
19	4	—	—	—	—	—	—	—	—	4
22	1	—	5	3	18	1	1	4	—	33
23	5	—	3	4	6	—	1	6	4	29
25	1	—	—	—	—	—	—	—	—	1
27	—	2	17	10	2	3	2	18	7	61
28	—	—	—	1	—	2	—	2	—	5
Wade	39	126	4	16	—	3	1	17	—	206
Stewart	—	—	24	—	3	—	9	14	2	52
Hempston	1	3	23	27	—	3	1	14	4	76
Alli	4	—	13	6	1	—	2	8	—	34
Coleman	—	—	5	3	1	1	—	1	3	14
Murphy	—	—	2	3	2	1	3	5	3	19
Doyle	—	—	—	4	—	2	1	3	—	10
John	2	1	2	3	4	1	1	5	—	19
Woodbury	1	3	—	—	6	—	—	1	—	11
Coghlan	—	—	—	—	—	—	4	1	—	5
Gerrard	2	3	—	1	—	—	—	—	—	6
Untyped	12	10	10	7	13	5	11	25	19	112
Total	285	163	189	158	71	39	150	221	65	1,341

* We are indebted to Dr. W. Gilmour for sending us these strains which were isolated in Auckland, New Zealand.

Griffith's types 7, 16, 20 and 21 are not of group A.

No strain was isolated of Griffith's type 3, 13, 14, 15, 18, 24, 26, 29 or 30 or of type "Hooper" or "Krone" established by Rudd, White and Ward (1939) in Sydney.

excised tonsils and surgical lesions; types "Wade", 11, 12, 17 and 2, however, were not uncommon in surgical lesions. Of the "surgical" types, neither type "Stewart" nor type 27 was found in scarlet fever, and types 6, "Hempston" and "Alli" were found in only 11 out of 285 scarlatinal patients. It is possible that in these 11 cases more than one type was present on the plate, and the single colony selected was not the cause of the attack of scarlet fever.

TABLE 2.

The distribution, among certain of the serological types, of the strains found in Melbourne in scarlet fever, excised tonsils or surgical lesions.

		"Scarlatinal" types.	"Surgical" types.
Source.	No. of typed strains.	Types 2, 17, Wade, 11.	Types 6, Stewart, Hempson, 27, Alli.
Scarlet fever	273	240 (88 p.c.)	11 (4 p.c.)
Excised tonsils	179	27 (15 p.c.)	115 (64 p.c.)
Surgical lesions	196	51 (26 p.c.)	92 (47 p.c.)

TABLE 3.

The sources, clinical distribution and characters of the new serological types represented in our collection.

Type.	No. of strains.	Source.	Distribution.	Remarks.
"Wade"	206	Rudd, White and Ward (1939), Sydney: scarlet fever.	In 5 p.c. of patients with scarlet fever in Sydney (Rudd, White and Ward (1939), 10 p.c. in Melbourne, 82 p.c. in Adelaide. Also in excised tonsils and surgical lesions.	Type "Wade" is closely related serologically to type 9; it is related serologically to type "Stewart" also, but differs from this type in fermenting starch.
"Stewart"	52	Dr. Hilda Gardner, Melbourne: pus.	In excised tonsils and surgical lesions; not isolated in scarlet fever.	Type "Stewart" is related serologically to types 9 and "Wade"; it differs from these types in not fermenting starch. It differs from all other types in not forming hydrogen peroxide. (See text, paragraph (f)).
"Hempston"	76	Melbourne: excised tonsils.	In excised tonsils and surgical lesions; in 4 patients with scarlet fever.	
"Alli"	34	Miss H. Butler, Melbourne: vaginal swab of patient with puerperal pyrexia.	In excised tonsils and surgical lesions; in 4 patients with scarlet fever.	Type "Alli" is related serologically to types 8 and 10. It differs from type 8 in fermenting cellobiose and from type 10 in its growth in serum-broth and serum-peptone-agar.
"Coleman"	14	Miss H. Butler, Melbourne: vaginal swab of patient with puerperal pyrexia.	In excised tonsils and surgical lesions; not isolated in scarlet fever.	Type "Coleman" is related serologically to types 8 and 10 from which it differs in fermenting starch and cellobiose. It is the only type which gives compact colonies in serum-peptone-agar but no opalescence in serum-broth; it is, therefore, the only exception which we have met to the presumptive test for group A streptococci of Ward and Rudd (1938).
"Murphy"	19	Dr. Hilda Gardner, Melbourne: nasal swab.	In excised tonsils and surgical lesions; not isolated in scarlet fever.	Type "Murphy" is related serologically to type 28 from which it differs in not fermenting starch and in its growth in serum-peptone-agar and serum-broth.
"Doyle"	10	Melbourne: throat swab.	In excised tonsils and surgical lesions; not isolated in scarlet fever.	Type "Doyle" is related serologically to type 28 from which it differs in not fermenting starch and in its growth in serum-peptone-agar and serum-broth.
"John"	19	Melbourne: scarlet fever.	In scarlet fever, excised tonsils and surgical lesions.	
"Woodbury"	11	Miss H. Butler, Melbourne: vaginal swab of patient with puerperal pyrexia.	In puerperal fever, scarlet fever and in one surgical lesion.	
"Coghlan"	5	Melbourne: throat swab.	In throat swabs from healthy subjects and in one patient with tonsilitis.	
"Gerrard"	6	Rudd, White and Ward (1939), Sydney: scarlet fever.	In 3 p.c. of patients with scarlet fever in Sydney (Rudd, White and Ward, 1939); rarely in scarlet fever in Melbourne and Adelaide.	

Table 2 emphasizes this broad difference in the distribution of the streptococci of these two groups in Melbourne. Common "scarlatinal" types readily caused either scarlet fever or pyogenic lesions, whereas the common "surgical" types rarely caused scarlet fever.

The origin and distribution of the local types not included in Griffith's types. Table 3 shows the sources and the clinical distribution of the local serological types. None is serologically identical with any of Griffith's types, although some were distinguished from some of Griffith's types only with difficulty by absorption of the type agglutinating sera. Sometimes differences in growth in serum-peptone-agar and serum broth (Ward and Rudd, 1938) or in

fermentation reactions pointed to serological differences in closely-related types. These reactions are shown in Table 4, which extends and corrects the table previously published (Keogh and Simmons, 1940). We suggested that type "Hale" was probably identical with Griffith's type 23; reciprocal absorption tests confirmed this. Type 23 (Griffith's strain "Barts 102") is serologically and culturally distinct from type 17 (Griffith's strain "Beatty"). As a result of experience with larger numbers of strains, other corrections have been made in the table previously published. Type 10 does not ferment starch. We now have strains of type 9 which appear as the rough coherent colony of Griffith (1934) or the "F" variant of Ward and Lyons (1935) and of Ward and Rudd (1938). This colony type differs from the "mucoid" type previously seen by growing as a "compact" colony in serum-peptone-agar instead of as a "feathery" colony.

TABLE 4.

The differential reactions of group A streptococci, including the new serological types tentatively established in Melbourne and Sydney.

Types isolated in Australia.	Colony in serum peptone agar.	Opalescence in serum broth.	Fermentation of Mannitol.	Starch.	Cellobiose.	Griffith's type strains.
2, 9	F	+	—	+	+	2, 9, 28
"Coghlan"	F	—	—	+	—	
1	F	—	—	+	+	1
6	F	—	+	—	(—)	6, 17
10, "Doyle"	F	—	—	—	—	15, 10
·	F	+	—	—	+	13
5, 12, 17, 19, 27, "Hempston", "Murphy"	F	—	—	—	+	3, 5, 11, 14, 18, 19, 24, 26, 29, 30
4, 28, "Wade", 9	C	+	—	+	+	4
23	C	+	+	—	(—)	23
11, 12, 27, "Stewart", "Alli", "John", "Gerrard"	C	+	—	—	+	12, 25, 27
22, 8, "Hooper"	C	+	—	—	—	8, 22
"Krone", "Woodbury"	C	+	—	+	(—)	·
"Coleman"	C	—	—	+	+	·

(—) These strains may ferment cellobiose from the fifth day.
F = feathery colony. C = compact colony.

Type distribution of strains isolated from patients with scarlet fever in various countries. Table 5 shows the predominant types of *Streptococcus pyogenes* isolated from patients with scarlet fever in various countries. In London types 1, 2, 3 and 4 have been responsible for most cases of scarlet fever for the past 13 years; the order of these four types has varied at different times. In Edinburgh in 1933 type 5 was found more often than types 3, 1, 2 or 4. In 1938, however, type 5 was not found at all; type 1 predominated and types 4, 2 and 3 were

TABLE 5.

The predominant types of Streptococcus pyogenes *isolated from patients with scarlet fever in various countries.*

Authority.	Predominant scarlet fever types.
Ministry of Health (1937), London, 1926–36	1, 2, 3, 4
Griffith (1934), England	1, 2, 3, 4
Green (1937), Edinburgh, 1933	5, 3, 1, 2, 4, "C"
Kodama *et al.* (1937), Tokyo, 1936–7	1, 2, 3, 4
Kodama *et al.* (1938), Tokyo, 1937–8	8, 4, 2, 10
Gundel and Wustenberg (1937), Gelsenkirchen	4, 3, 5, 1, 2
White *et. al.* (1939), Sydney, 1936	17, 1, 4, 6, "Wade"
White *et al.* (1939), Sydney, 1938	11, 4, 17, "Wade", 1
Neisser (1939), London	1, 2, 4, 3
Bailey (1939), Chicago, 1934–5	2, 3, 6
de Waal (1940), Edinburgh, 1937–9	1, 2, 3, 4
Present series, Melbourne, 1938	2, 17, "Wade", 11
Present series, Adelaide, 1938–9	"Wade"

common. In Tokyo in 1936–37 the common types were 1, 2, 3 and 4, but, a year later, types 8 and 10 were prominent. In a German town type 5 was found with types 1, 2, 3 and 4. In Sydney types 17, 11, 4, 6 and a type not included in Griffith's classification, type "Wade", were commonest, whereas types 2 and 3 were not found at all; two years later type 6 had disappeared and type 11 was found more often than any other type. In Chicago in 1934–35 types 2, 3 and 6 were commonest. In Melbourne for the past three years types 2, 17, "Wade" and 11 have predominated, whereas type 1 has never been found. In Adelaide type "Wade" has been isolated from 82 p.c. of scarlatinal patients.

The sudden appearance of type 8 in Tokyo and of type 11 in Sydney as epidemic types and the predominance of type "Wade" in Adelaide are interesting. It is not known if type "Wade" or other local types occur outside Australia and New Zealand.

SUMMARY.

1,341 strains of *Streptococcus pyogenes* (Group A) from various human sources have been examined; 1229 have been serologically typed, 112 remain untyped.

Some of the technical methods used in the isolation and typing of *Str. pyogenes* are discussed.

The same serological types were common to tonsils removed at operation and surgical lesions. These "surgical" types were rare in scarlet fever; the types common in scarlet fever, however, were found in 26 p.c. of surgical lesions.

The origin, distribution and characters of 13 new serological types, tentatively established in Sydney or Melbourne, are stated.

The type distribution of scarlatinal strains in various countries is discussed.

REFERENCES.

Bailey, J. H. (1938): Amer. J. Hyg., 29, p. 107.
de Waal, H. L. (1940): J. Hyg., Camb., 40, p. 172.
Fuller, A. T. and Maxted, W. R. (1939a): Brit. J. exp. Path., 20, p. 177.
Fuller, A. T. and Maxted, W. R. (1939b): J. Path. Bact., 49, p. 83.
Green, C. A. (1937): J. Hyg., Camb., 37, p. 318.
Griffith, F. (1934): J. Hyg., Camb., 34, p. 542.
Gundel, M. and Wustenberg, J. (1937): Zbl. Bakt., I Abt., Orig., 138, p. 325.
Hadley, F. P. and Hadley, P. (1940): J. Bact., 39, p. 21.
Keogh, E. V. and Kelsey, H. (1939): Med. J. Austral. 1, p. 100.
Keogh, E. V., Macdonald, I., Battle, J. and Puckey, M. C. (1939): *Ibid.*, 1, p. 792.
Keogh, E. V., Macdonald, I., Battle, J., Simmons, R. T. and Williams, S. (1939a): J. Hyg., Camb., 39, p. 664.
Keogh, E. V., Macdonald, I., Battle, J., Simmons, R. T. and Williams, S. (1939b): Brit. Med. J., 2, p. 1036.
Keogh, E. V. and Simmons, R. T. (1940): J. Path. Bact., 50, p. 137.
Kodama, T., Chiku, Y., Kodaira, T. and Kodama, A. (1937): Kitasato Arch. exp. Med., 14, p. 245.
Kodama, T., Ozaki, M., Nishiyama, S. and Chiku, Y. (1938): *Ibid.*, 15, p. 162.
Macdonald, I., Simmons, R. T. and Keogh, E. V. (1940): Med. J. Austral., 1, p. 849.
Ministry of Health, London, Annual Rep. of Chief Med. Off., 1937: p. 214.
Neisser, H. (1939): J. Path. Bact., 48, p. 55.
Pauli, R. H. and Coburn, A. F. (1937): J. exp. Med., 65, p. 595.
Rudd, G. V., White, C. and Ward, H. K. (1939): Austral. J. exp. Biol., 17, p. 25.
Sherman, J. M. (1937): Bact. Rev. 1, No. 1, pp. 11, 20.
Simmons, R. T. and Keogh, E. V. (1940): Austral. J. exp. Biol., 18, p. 151.
Simmons, R. T. and Wilson, H. (1941): Med. J. Austral., 1, p. 74.
Ward, H. K. and Lyons, C. (1935): J. exp. Med., 61, p. 515.
Ward, H. K. and Rudd, G. V. (1938): Austral. J. exp. Biol., 16, p. 181.
White, C., Rudd, G. V., Ward, H. K., Wilson, F. H. and Symington, N. J. (1939): Med. J. Austral., 1, p. 96.

OBSERVATIONS ON THE ROLE OF SODIUM CHLORIDE IN THE AGGLUTINATION OF BACTERIA BY ANTIBODY[1]

by A. E. PLATT

(From the Department of Pathology, Cambridge, the Department of Bacteriology, University of Adelaide, and the Institute of Medical and Veterinary Science, Adelaide).

(Accepted for publication 28th November, 1940.)

The classical "two-stage" hypothesis of the mechanism of agglutination relegated the action of the electrolyte to the second stage of the reaction, but recent work shows that electrolyte also exerts a powerful influence on the first stage. Duncan (1934, 1937) showed that the zone of maximal antibody fixation approximated to the salt optimum, and that the antiserum/suspension ratio of the antibody optimal mixture varied with the concentration of sodium chloride. With a smooth strain of *Br. abortus* (Pandit and Wilson, 1932) and a corresponding antiserum the author (Platt, 1938) found that the serum/suspension optimal ratios remained constant for a wide range of salt concentrations. Using a similar system, Miles (1939) demonstrated a slight shift in the serum/suspension ratios with change in salt concentration. To elucidate these discrepancies investigations were made on the action of sodium chloride in two systems, viz. (i) suspensions of *Br. abortus* and the serum of a cow with brucellosis; (ii) an H-suspension of *Bact. typhosum* and a *Bact. stanley* antiserum.

EXPERIMENTAL WORK.

A. *Preparation of Reagents.*

Suspensions. Two suspensions of *Br. abortus* were used, viz. (i) the original suspension (see Platt, *loc. cit.*), and (ii) a similar suspension prepared from the same strain one year later. This strain was still salt-stable, though not entirely dependent upon CO_2 for growth. Before use in the electrophoresis experiments, the original suspension was washed three times in distilled water without observable effect upon the salt-antigen-antibody relationships.

A 24-hour culture of *Bact. typhosum* (No. 302, Cambridge) was washed off in distilled water and killed by heating at 55° C. for 30 minutes. After three washings in distilled water the organisms were suspended in 0·25 p.c. formalin and the suspension was dialysed for 48 hours.

Antisera. The original Brucella and dialysed *Bact. stanley* antisera were used. Against a *Bact. typhosum* suspension (500×10^6 org. per ml.) the *Bact. stanley* antiserum gave complete agglutination at a final dilution of 1/10,240.

Electrolyte. Pure sodium chloride (A.R.) was made up in doubly distilled water and will be referred to as "salt".

B. *The Effect of Salt upon the Agglutination of* Br. abortus *by the homologous Antiserum.*

The results of most of these observations were recorded in a previous paper. Two additional experiments are recorded here.

The effect of salt upon the combination of antibody and antigen. The amount of antibody remaining free after absorption of a series of antiserum dilutions by a $1{,}000 \times 10^6$ org. per ml. suspension at various salt concentrations was titrated against a light suspension (500×10^6 org. per ml.) in M/14. At a 1/175 antiserum dilution the maximum amount of antibody was absorbed in the presence of 2M/7 salt, or the salt optimum for that particular serum/suspension ratio. When the test was repeated in the presence of normal serum the maximal antibody absorption occurred with M/14 salt which was also the salt optimum. With higher dilutions of antiserum the amount of free antibody was too small for titration. The results support Duncan's conclusion that the zone of maximal antibody absorption approximates to the salt optimum.

[1] The results presented here were submitted for the Ph.D. degree of the University of Cambridge. Owing to the state of emergency it has been necessary to present them here in an abbreviated form.

The influence of age of strain on the effect of salt upon the antigen and antibody optima. In the determination of all optima a modification of the illuminated water bath described by Miles (1933) was used. In this bath provision was made for maintaining (i) the tubes constantly under observation, (ii) adequate convection currents in the tubes. The details may be seen in Fig. 1.

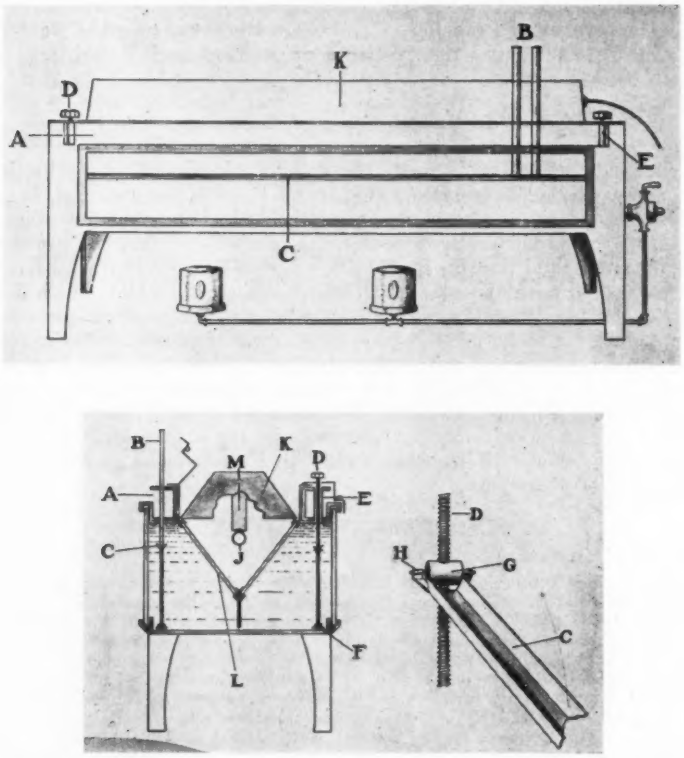


Fig. 1. Illuminated water bath used in the determination of various optima.

In a series of constant antigen titrations at M/7, M/14 and M/28 salt, the *antibody* optimal serum/suspension ratio remained constant over a wide range of antigen concentrations at M/28 salt only. At higher salt concentrations the optimum moved to higher serum/suspension ratios with dilute suspensions (less than 4×10^9 org. per ml.). The *antigen* optimum showed a slight movement to higher serum/suspension ratios with the two higher concentrations of salt.

C. *The Effect of Salt upon the Agglutination of* Bact. typhosum *by* Bact. stanley *Antiserum.*

The effect of various salt concentrations on the end-point of agglutination. The *Bact. stanley* antiserum was titrated at various salt concentrations against a suspension (500×10^6 org. per ml.) of *Bact. typhosum.* Readings were made after two hours at 55° C. and overnight in the refrigerator. The highest end-point titre was obtained at M/28 and M/56 salt. With less salt there was a marked falling-off in titre, and M/896 was about the minimum concentration capable of producing agglutination under the test conditions.

The effect of salt upon the antigen optimum. An *antibody* optimum was not obtained. Experiment showed that it lay in the zone of gross antibody excess. As found by Duncan the *antigen* optimum was sensitive to salt. At M/7 salt, 3×10^9 organisms contained in 1 ml.

TABLE 1.

The salt optima of a number of mixtures of Bact. typhosum *suspension and* Bact. stanley *antiserum.*

Suspension No. organisms per ml. × 10^6	Antiserum dilutions.	Ratio No. organisms to 1 ml. antiserum × 10^{11}	Salt concentrations. M/14	M/28	M/56	M/112	M/224	M/448	M/896
2,000	1/400	8				+			
	1/800	16			+	+			
	1/1600	32			+				
	1/3200	64		+					
	1/6400	128		+					
	1/12800	256			+				
1,000	1/400	4				+			
	1/800	8			+				
	1/1600	16		+	+				
	1/3200	32		+					
	1/6400	64		+					
	1/12800	128		+	+				
500	1/400	2					+		
	1/800	4				+			
	1/1600	8			+				
	1/3200	16		+					
	1/6400	32		+					
	1/12800	64		+	+				
250	1/400	1			+				
	1/800	2		+					
	1/1600	4		+					
	1/3200	8		+					
	1/6400	16		+					
	1/12800	32			+				

+ indicates the position of the salt optimum.

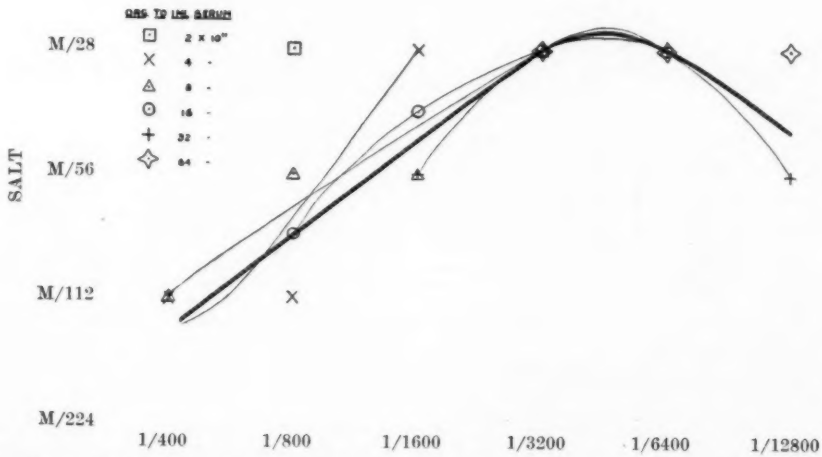


Fig. 2. Showing change of salt optimum with increasing antiserum dilution for various suspension/serum ratios of *Bact. typhosum* and *Bact. stanley* antiserum. Mean curve in heavy lining.

flocculated optimally with an equal volume of antiserum diluted 1/400—a ratio of 12×10^{11} organisms to 1 ml. antiserum. At a serum dilution of 1/3,200 in the same salt concentration, 75×10^{7} organisms gave optimal flocculation—a ratio of 24×10^{11} organisms to 1 ml. antiserum.

The salt optima of serum/suspension mixtures. The results are given in Table 1. From these results the salt optima of a series of suspension/serum ratios for various antiserum dilutions were calculated. In Fig. 2 these values have been plotted against antiserum dilutions. The smooth curves for mixtures with suspension/serum ratios equivalent to, and greater than 8×10^{11} organisms per ml. of antiserum approximate to a general smooth curve which may be taken as illustrating the behaviour of a wide range of suspension/serum ratios. Over a relatively wide range of antiserum dilutions the optimal salt concentration is approximately inversely proportional to the antiserum concentration. With higher degrees of sensitization the compounds behave differently.

In view of the known relationship between the zone of maximal antibody fixation and the salt optimum, the effect of "correction" of the salt concentration upon the *antigen* optimum was noted. Preliminary experiments suggested that a mixture containing 3×10^{9} organisms per ml. and antiserum diluted 1/400 was the *antigen* optimal suspension/serum ratio. The salt optimum of this mixture as well as of others prepared from a series of twofold dilutions of antiserum and of suspension, thus possessing the same suspension/serum ratio, was determined. The results are given in Table 2. When the *antigen* optimum was again determined with the salt concentration corrected for each antiserum dilution the optimum remained constant over a wide range of antiserum dilutions (Table 3). Because of the high dilutions of antiserum, which for practical reasons it was necessary to use, the degree of antibody absorption could not be studied.

TABLE 2.

The salt optimum of a series of mixtures of Bact. typhosum *and* Bact. stanley *antiserum.*

Suspension No. organisms per ml. $\times 10^{6}$	Antiserum dilutions.	Ratio No. organisms to 1 ml. antiserum $\times 10^{11}$	Salt concentration M/14	M/28	M/56	M/112	M/224	M/448	M/896	Salt Optimum.
3,000	1/400	12	+	+	x——x		±	tr.	f.tr.	M/84
1,500	1/800	12	+	x——x		+	±	tr.	f.tr.	M/42
750	1/1600	12	+	x	+	±	±	tr.	v.f.tr.	M/28
375	1/3200	12	+	x	+	±	v.f.tr.	—	—	M/28
187·5	1/6400	12	+	x——x		±	—	—	—	M/42
93·75	1/12800	12	—	—	x	—	—	—	—	M/56

x indicates position of optimum.

TABLE 3.

The effect of correction of salt concentration on the antigen optimal ratio of Bact. typhosum *suspension and* Bact. stanley *antiserum.*

Antiserum dilutions.	Suspension. No. organisms $\times 10^{6}$ 3,000	1,500	750	375	187·5	93·75	Salt concentrations.
1/400	x	+	+	+	+	+	M/84
1/800	+	x	+	+	+	+	M/42
1/1600	±	+	x	+	+	tr.	M/28
1/3200	f.tr.	tr.	±	x	tr.	f.tr.	M/28
1/6400	—	—	±	+	x	tr.	M/42
1/12800	—	—	—	—	v.f.tr.	x	M/56

x indicates position of optimum.

D. *Electrophoresis.*

This section of the work was undertaken to correlate, if possible, the results of the observations detailed above and in the previous paper with the effect of salt upon the electrokinetic potential ζ and the charge density σ.

Technique. The moving boundary method proved entirely satisfactory with both suspensions. The type of apparatus used (see Fig. 3) was a modification of that described by Northrop and Cullen (1921–22) and was made by Mr. R. H. Oliphant, of the Physics Department, University of Adelaide. The inclusion of terminals (D) in the lower end of each arm of the

U-tube made possible an accurate determination of the potential drop through the instrument. Current was supplied to the zinc electrodes from dry batteries at 120 volts.

The ζ-potential was calculated from the Helmholtz-Lamb equation relating velocities and potential. The charge density σ was then calculated from the formula relating charge density and the ζ-potential (see Abramson, 1934).

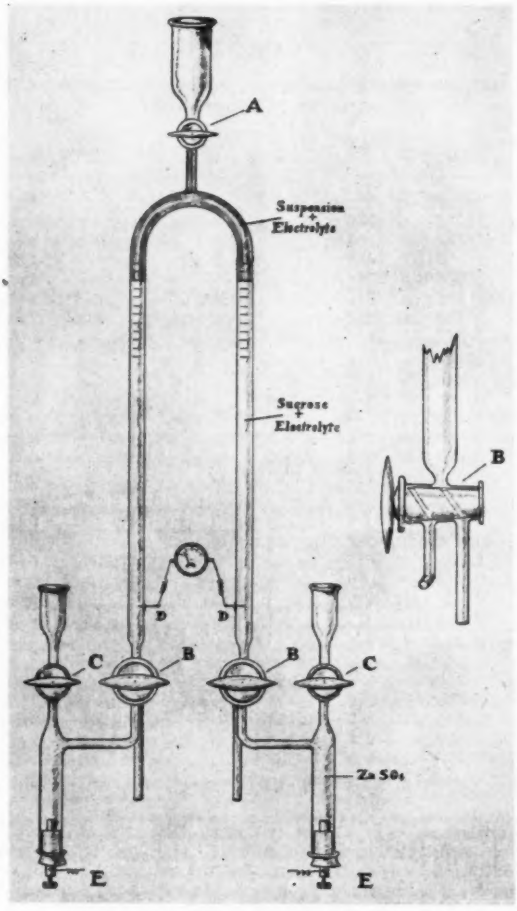


Fig. 3. Electrophoresis apparatus.

In preliminary experiments phosphate buffer was used to maintain the pH constant at 7·0. The buffer, however, masked the true effect of the salt. This has been noted by other workers (Mr. G. Adair, private communication), and the buffer was omitted. The pH of the various mixtures which were prepared ten minutes before being submitted to electrophoresis varied between 6·8 and 7·2.

The ζ-potential of Br. abortus *and* Bact. typhosum. In a large series of experiments it was found that the electrokinetic potential of the organisms suspended in distilled water varied with the density of the suspension—the denser the suspension the lower the electrokinetic potential of the organisms. Variation in the CO_2 content of the water was not responsible.

The effect of normal (cow) serum and immune serum on the ζ-potential of Br. abortus. The results, given in Table 4 (see also Fig. 4), show that (i) serum proteins reduced the ζ-potential; (ii) a point of "saturation" was reached at 1/350 serum; (iii) in the presence of salt normal serum had little effect upon the potential; (iv) with antiserum the potential was lowest in the presence of salt at the *antibody* optimum; (v) in the lower antiserum dilutions the potential was often higher in the presence of salt than in its absence. The antiserum produced similar results with suspensions of 2,857 × 10^6 and 500 × 10^6.

TABLE 4.

The effect of normal serum and antiserum upon the electrokinetic potential of Br. arbortus (1,000 × 10^6 per ml.).

		Normal serum.					Antiserum.		
Salt concentration.	Serum dilution.	Velocity μ/sec./volts/cm.	Potential mv.	Charge density E.S.U.	Mean potential mv.	Mean density E.S.U.	Velocity μ/sec./volts/cm.	Potential mv.	Charge density E.S.U.
Nil	1/87·5	1·555	19·6				0·621	7·8	
	1/175	1·382	17·4				0·962	12·1	
	1/350	1·382	17·4				1·180	14·9	
	1/700	1·858	23·4				1·330	16·8	
	1/1400	2·395	30·2				1·442	18·2	
	1/2800	2·555	32·2				2·020	25·4	
	nil	2·095	26·4				2·095	26·4	
M/56	1/87·5	0·522	6·6	616			0·698	8·8	827
	1/175	0·544	6·9	643			0·551	6·9	648
	1/350	0·639	8·1	756			0·542	6·8	640
	1/700	0·617	7·8	729	7·3	685	0·323	4·7	440
	1/1400	0·603	7·6	715			0·493	6·2	583
	1/2800	0·548	6·9	649			0·527	6·6	622
	nil	0·670	8·5	794			0·670	8·5	794
M/28	1/87·5	0·466	5·9	779			0·562	7·1	937
	1/175	0·443	5·6	738			0·487	6·1	813
	1/350	0·419	5·3	700			0·356	4·5	593
	1/700	0·513	6·5	856	5·7	763	0·324	4·1	540
	1/1400	0·476	6·0	794			0·357	4·5	595
	1/2800	0·400	5·0	668			0·462	5·8	772
	nil	0·399	5·0	668			0·399	5·0	667
M/14	1/87·5	0·356	4·5	840			0·448	5·7	1,060
	1/175	0·360	4·5	849			0·428	5·4	1,013
	1/350	0·350	4·4	827			0·260	3·3	612
	1/700	0·310	3·9	733	4·0	748	0·243	3·1	573
	1/1400	0·310	3·9	733			0·328	4·1	776
	1/2800	0·295	2·7	508			0·404	5·1	955
	nil	0·335	4·2	791			0·333	4·2	791

A 1/700 dilution of antiserum constitutes with 1,000 × 10^6 organisms the *antibody* optimum.

The action of antiserum was modified by the addition of normal serum in a final dilution of 1/350. In antiserum dilutions greater than 1/175 the potential of organisms suspended in water, M/56 and M/14 salt, was approximately the same. With more antiserum present the potential was higher with the organisms suspended in salt than in water.

With organisms which had been sensitized in the absence of salt by overnight contact in the cold with antiserum and from which excess antibody had been removed by washing, the ζ-potential of highly sensitized organisms showed no tendency, when suspended in salt, to exceed that of the same organisms suspended in water.

The effect of normal (rabbit) serum and immune serum on the ζ-potential of Bact. typhosum. The results, given in Table 5, show that (i) both normal serum and antiserum reduced the ζ-potential of organisms suspended in water and very dilute salt; (ii) normal serum had no effect on the potential in concentrations of salt above M/56.

The ζ-potential of collodion particles coated with serum proteins. Collodion particles, prepared by the method of Loeb (1922), were suspended in distilled water to give an opacity equivalent to that of a 2 × 10^9 *Bact. coli* suspension. Equal volumes of this suspension and of serum diluted 1/87·5 were mixed, and ten minutes later the electrokinetic potential of the particles was determined (see Table 6).

TABLE 5.

The effect of normal serum and antiserum on the electrokinetic potential of Bact. typhosum (500×10^6 per ml.).

Salt concentration.	Serum dilution.	Normal serum. Velocity μ/sec./ volts/cm.	Potential mv.	Charge density E.S.U.	Antiserum. Velocity μ/sec./ volts/cm.	Potential mv.	Charge density E.S.U.
Nil	1/400	0·428	5·4		0·649	8·3	
	1/800	0·524	6·6		0·769	9·7	
	1/1600	0·647	8·2		0·968	12·2	
	1/3200	0·965	12·2		1·102	13·9	
	1/6400	1·032	13·0		1·331	16·8	
	1/12800	1·118	14·1		1·315	16·6	
	nil	1·368	17·3		1·368	17·3	
M/224	1/400	0·588	7·4	110	0·632	8·0	119
	1/800	0·632	8·0	118	0·725	9·1	136
	1/1600	0·663	8·4	124	0·739	9·3	139
	1/3200	0·648	8·2	121	0·884	11·2	166
	1/6400	0·894	11·3	168	0·848	10·7	159
	1/12800	0·894	11·3	168	0·916	11·5	172
	nil	1·112	14·0	210	1·112	14·0	210
M/112	1/400	0·652	8·2	172	0·551	6·9	146
	1/800	0·684	8·2	172	0·551	6·9	146
	1/1600	0·684	8·2	172	0·627	7·9	166
	1/3200	0·835	10·5	222	0·640	8·1	170
	1/6400	0·898	11·3	239	0·632	8·0	168
	1/12800	0·981	12·4	262	0·753	9·5	200
	nil	0·855	10·8	228	0·855	10·8	228
M/56	1/400	0·585	7·4	692	0·440	5·5	519
	1/800	0·572	7·2	676	0·466	5·9	550
	1/1600	0·482	6·1	570	0·466	5·9	550
	1/3200	0·520	6·6	614	0·478	6·0	560
	1/6400	0·533	6·7	630	0·602	7·6	712
	1/12800	0·663	8·4	785	0·542	6·8	640
	nil	0·685	8·6	811	0·685	8·6	811
M/28	1/400	0·572	7·2	956	0·264	3·3	441
	1/800	0·550	6·9	920	0·307	3·9	513
	1/1600	0·613	7·7	1,025	0·377	4·7	628
	1/3200	0·500	7·6	1,006	0·334	4·2	559
	1/6400	0·613	7·7	1,025	0·377	4·7	628
	1/12800	0·676	8·5	1,131	0·460	5·8	769
	nil	0·562	7·1	941	0·562	7·1	941

TABLE 6.

The potential of collodion particles coated with serum proteins.

Salt concentration.	Serum 1/87·5.	Velocity μ/sec./volts/cm.	Potential millivolts.	Charge density E.S.U.
Nil	Nil	1·920	24·2	
M/112	Nil	4·140	52·2	1,305
M/56	Nil	3·175	40·1	4,160
M/28	Nil	2·745	34·6	4,950
M/14	Nil	2·181	27·7	5,460
M/112	normal	0·796	10·1	212
M/56	normal	0·673	8·5	794
M/28	normal	0·558	7·0	932
M/14	normal	0·599	7·6	1,416
M/112	antiserum	0·820	10·3	218
M/56	antiserum	0·568	7·4	688
M/28	antiserum	0·515	6·5	862
M/14	antiserum	0·360	4·5	849

DISCUSSION.

In the process of agglutination by antibody the bacterial cells become aggregated into clumps which, by virtue of their mass, overcome the thermodynamic forces holding them in suspension and they settle out. The phenomenon is generally explained in terms of one or other of the two major hypotheses, viz.: (i) the "two-stage" hypothesis and (ii) a modification of Marrack's lattice theory of precipitation.

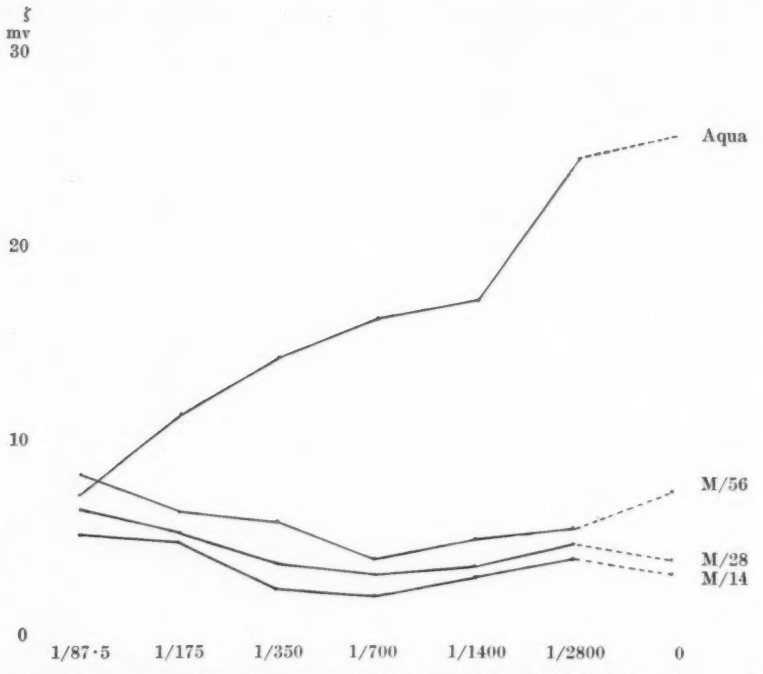


Fig. 4. Showing effect of antiserum on the electrokinetic potential of *Br. abortus*. Suspension = 1,000 × 10^6 organisms per ml.

The "two-stage" theory states that, in the process of sensitization, the organism absorbs globulin which becomes denatured. This produces a change in the bacterial surface from hydrophile to hydrophobe with consequent sensitivity to the flocculating action of electrolyte (see Northrop and de Kruif, 1921–22; Eagle, 1930). The "coated" organisms, like any denatured protein, then flocculate non-specifically.

This theory depends for its validity upon two points, viz.: (i) "denaturation" of the surface globulin film and (ii) sensitization producing a hydrophile to hydrophobe change. The term "denaturation" usually implies certain definite alterations in the structure of the protein molecule which render it hydrophobe. This process is irreversible. The change, if any, which occurs in the antibody molecules, however, is reversible to some extent. Antibody fixed by antigen can be "eluted" under favourable conditions, and appears unaltered by the procedure. Astbury (quoted by Marrack, 1938) examined the globulin in two specific precipitates from antipneumococcal sera by X-ray analysis and found no evidence that the proteins had changed from the globular form.

Certain changes, which cannot be placed under the heading "denaturation" may occur in protein molecules. Abramson (1934) defined this process as "surface denaturation", and it is indicated by a slight shift in the isoelectric point of the protein. This still implies a change from hydrophile to hydrophobe. Sensitization is not always accompanied by this change in surface character. Mudd and Mudd (1927) showed that acid-fast organisms changed from a hydrophobe to a hydrophile state in the process, "wettability" by oil and by water being the criterion of the two states respectively.

Marrack's lattice theory, modified to suit the requirements of a system wherein the antigen molecules constitute part of a cell surface, implies the specific linking of organisms by antibody molecules (see Topley and Wilson, 1936). Marrack (1938) does not accept this view and believes that the second stage of the reaction is non-specific in character. It is too early to decide between these two hypotheses; nor need we reject one entirely in subscribing to the other.

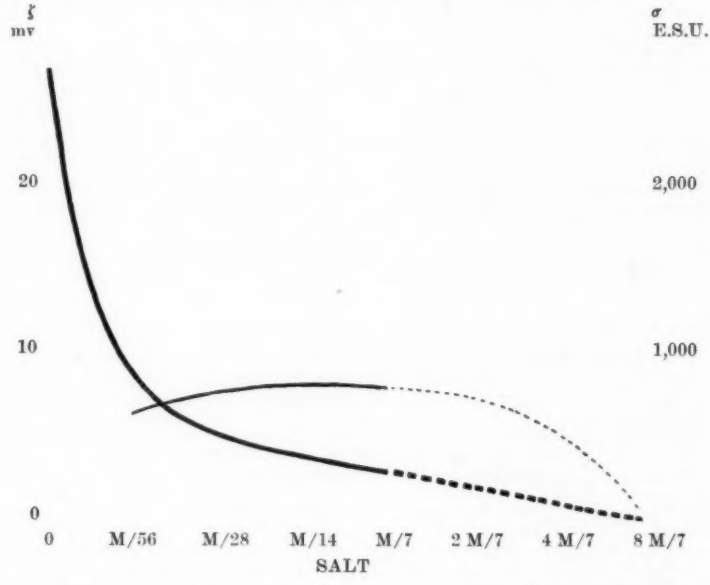


Fig. 5. The ζ- and σ- salt concentration curves for *Br. abortus*. Complete line indicates observed values. Broken line indicates calculated values.
——— ζ- potential. ——— $= \sigma$.

It is convenient to discuss the phenomenon as occurring in two stages, viz.: (i) the fixation of antibody; (ii) the formation of aggregates. At the moment of mixing, the cells and antibody molecules are surrounded by "ionic atmospheres" characteristic of the surface responsible for their existence. The algebraical sum of these is a measure of the force preventing fixation of antibody. As the cell surface becomes covered with protein the force becomes modified until at the point of complete surface "saturation" it approaches the algebraical sum of those forces existing at two protein surfaces. The forces keeping two cells apart also vary with the degree of sensitization. With light sensitization the charging process approximates to that of free cells, with maximum sensitization to that of protein.

In this hypothesis emphasis has been laid on electrokinetic phenomena as the cause of stability of a bacterial suspension. Early observers (see Northrop and de Kruif, *loc. cit.*) noted this and studied the effect of electrolyte upon the stability of suspensions of sensitized and unsensitized cells. These observations are applicable only to the second stage of agglutination. Duncan's observations indicated that similar effects were operative in the first stage. For

technical reasons it was not possible in most instances to determine the ζ-potential at concentrations of salt greater than M/14. The rate of decrease of potential with increase in salt indicates that relatively high concentrations are required to reduce ζ to less than 1 mv. Extrapolation of the ζ-potential curve suggests that this point would be reached at M/7 salt (see Fig. 5). On this assumption the σ-concentration curve reaches a maximum between M/14 and M/7 salt and falls rapidly with further increase in salt concentration.

Similar information regarding antibody is lacking. Even with adequate quantities of pure antibody globulin technical difficulties would render the determination of ζ- and σ-concentration curves very difficult. In measuring the electric mobility of proteins, buffer solutions or dilute acids are invariably used to stabilize the potential gradient across the boundary. The effect of a single electrolyte, therefore, cannot readily be observed. The difference in potential between the free and serum-treated collodion particles indicates that protein was adsorbed by these particles. In the higher salt concentrations antibody was adsorbed to a greater degree than normal serum protein. In both cases increase in salt caused a progressive increase in σ. This departure from the form of the curve given by an *ideal* amorphous protein (see Abramson, *loc. cit.*) may indicate that the particle surface was not completely covered by protein, but more likely was due to a combination of factors, one of which was the mixture of proteins present. Support for the view that the charging process of antibody globulin is altered by high concentrations of salt is found in Table 3 of the previous paper. After exposure to 2 M/7 salt, antiserum agglutinated *Br. abortus* over a wider range of concentrations than when exposed to less salt.

If charge density constitutes an obstacle to fixation of antibody by the bacterial surface this process should occur freely in distilled water. The results given in Tables 4 and 5 show that this happened with both *Br. abortus* and *Bact. typhosum*. Increase in antiserum concentration produced a progressive decrease in the potential of the organisms. This was interesting because the *Bact. typhosum* system was of the H. type. In the presence of salt increase in antibody up to the *antibody* optimal amount also progressively reduced the potential; with more antibody the potential increased and might exceed that obtained at the same serum concentration in distilled water. Evidence available indicates that salt is responsible for this phenomenon. The potential-antiserum curves for distilled water and M/56 salt diverged in the zone of high sensitization when *Br. abortus* was sensitized in the absence of electrolyte. In concentrations of antibody greater than the *antibody* optimal amount high concentrations of salt are necessary (with low values for σ) for free fixation of antibody (see also previous paper).

At an antiserum dilution of approximately 1/350 the surface of *Br. abortus* ($1{,}000 \times 10^6$ per ml.) reached antibody "saturation" point (see Table 4). This does not mean that the entire surface of the organism was covered by a protein film; probably the receptor groups only. At a 1/700 dilution (*antibody* optimum for $1{,}000 \times 10^6$ organisms per ml.) the cell surfaces would be about half "saturated"; this should be the best condition for aggregation. Duncan (*loc. cit.*) found that the union of organisms in the aggregate was strongest at the *antibody* optimum. At antiserum concentrations in excess of "saturation" point, the number of bonds attaching antibody molecules to the cell surface is less than that required for firm union and, in the presence of electrolyte, many molecules probably break away shortly after making contact. This leaves the cell surface in a state of partial "saturation" with a potential higher than that existing at "saturation" point. As a compensation, a high salt concentration is necessary to reduce ζ and σ to negligible values.

Additional evidence of the influence of the surface charging process upon the fixation of antibody comes from the experiments with the second *Br. abortus* suspension. The serological optima of this suspension, prepared when the organism was probably undergoing an S $\rightarrow$ SR variation (see Miles, 1939), were sensitive to salt action. "Correction" of the salt concentration restored the original constancy of the optima.

Aggregation of the sensitized organisms should occur most readily at the point of half "saturation". Specific linking of the cells in this state results in a fully "saturated" surface with a minimal electrokinetic potential. The combination of these two factors produces the *antibody* optimum. The results given above support this view.

In view of the supposed difference in the distribution of the active antigenic groupings in the two systems investigated it was surprising to find such a marked degree of similarity in behaviour. All the *Bact. typhosum* mixtures had serum-suspension ratios less than the antibody optimal ratio. The salt optimum and ζ-concentration curves of this system correspond, therefore, with the similar curves for *Br. abortus* mixtures on the antigen-excess side of the *antibody* optimum.

In addition to any theoretical value attached to observations upon the rôle of salt in agglutination the findings have a practical application. Where a series of falling concentrations of antiserum is set up against a constant amount of bacterial suspension in 0·85 p.c. salt, the stabilizing forces vary from tube to tube. If this variation were due entirely to differences in antiserum concentration, the observed

result would give a true measure of the antibody content of the serum. The observations described above and those of Bier (1931) and Duncan (*loc. cit.*) indicate otherwise. At M/7 salt the maximum end-point titre was not reached with either system investigated here. This would be of no significance if all strains of a species and all corresponding antisera had the same "salt characteristics". Titrations of different sera at any salt concentration (within limits) would then give strictly comparable results. At present we have insufficient information on this point, and the behaviour of the Adelaide strain of *Br. abortus* indicates the probable existence of this type of variation.

Correction of salt concentration is important in the determination of the *antibody* and *antigen* optima, if constancy of these over a wide range of suspension and antiserum concentrations be accepted as a criterion of the true optimum. It seems likely also that variation in the serological optima with variation in salt concentration is an indication of an S → SR mutation in *Br. abortus*. This point needs further investigation.

SUMMARY.

The effect of salt upon the agglutination of *Bact. typhosum* by *Bact. stanley* antiserum was studied and the findings, together with those on *Br. abortus* recorded in a previous paper, are interpreted in the light of the results of electrophoresis experiments.

For each mixture of suspension and antiserum there is a concentration of salt which produces optimal flocculation. This concentration approximates to that at which maximal absorption of antibody occurs. The highest end-point titre is obtained with the salt concentration which is optimal for the smallest sensitizing dose of antibody.

The serological optima may show variation in the serum-suspension ratio with change in salt concentration. With *Br. abortus* this is probably an indication of an S → SR mutation.

Antiserum and normal serum proteins are adsorbed by organisms in the absence of salt, and produce a fall in the electrokinetic potential at the surface-water interface. In the presence of salt antiserum alone is absorbed in appreciable amounts which are dependent upon the relative concentrations of salt and antibody.

An hypothesis of the rôle of salt in agglutination is enunciated. The principal effect of salt is to reduce to some critical level the forces preventing (i) combination of antigen and antibody, and (ii) cohesion of the sensitized organisms.

Acknowledgment. I wish to thank Professor H. R. Dean for extending to me the hospitality of his Department and for continued interest during this investigation.

REFERENCES.

Abramson, H. A. (1934): "Electrokinetic Phenomena and their Application to Biology and Medicine", New York Chemical Catalogue Company. Monograph No. 66. Published by American Chemical Society.
Bier, O. G. (1931): C.R. Soc. Biol., Paris, 108, p. 511.
Duncan, J. T. (1934): Brit. J. exp. Path., 15, p. 23.
Duncan, J. T. (1937): *Ibid.*, 18, p. 108.
Eagle, H. (1930): J. Immunol., 18, p. 393.
Loeb, J. (1922–23): J. gen. Phys., 5, p. 109.
Marrack, J. R. (1938): Spec. Rep. Ser. med. Res. Coun., No. 230, London.
Miles, A. A. (1933): Brit. J. exp. Path., 14, p. 43.
Miles, A. A. (1939): *Ibid.*, 20, p. 63.
Mudd, S. and Mudd, E. B. H. (1927): J. exp. Med., 46, p. 173.
Northrop, J. H. and Cullen, G. E. (1921–22): J. gen. Phys., 4, p. 635.
Northrop, J. H. and Kruif, P. H. de (1921–2): *Ibid.*, 4, pp. 639, 655.
Pandit, S. R. and Wilson, G. S. (1932): J. Hyg., Camb., 32, p. 45.
Platt, A. E. (1938): Austral. J. exp. Biol., 16, p. 275.
Topley, W. W. C. and Wilson, G. S. (1936): "The Principles of Bacteriology and Immunity", 2nd Edit., London, Edward Arnold & Co.

THE INFLUENCE OF ANTERIOR PITUITARY EXTRACTS UPON THE RATE OF OXIDATION AND REDUCTION OF GLUTATHIONE IN TISSUES[1]

by A. H. ENNOR AND CHARLOTTE M. ANDERSON

(From the Baker Institute for Medical Research, Alfred Hospital, Melbourne).

(Accepted for publication 20th December, 1940.)

In a previous communication one of us (Ennor, 1939) showed that administration of anterior pituitary extracts to rabbits produced a definite diminution in the reduced glutathione (GSH) content of the liver. In addition, such treatment decreased the rate of oxidation of GSH in saline extracts prepared from liver tissue. Similar experiments with rats revealed that whilst a decrease in the GSH content of the liver was produced, no effect upon the oxidation mechanisms was discernible. This is possibly due to the rapid oxidation rate of GSH which was originally described by Hopkins and Elliott (1931–32) and was confirmed in our experiments. Many experiments were carried out using different procedures, but although results suggestive of an inhibition were obtained, they were in general inconclusive.

Continuing these lines of investigation, it was decided to determine whether the administration of anterior pituitary extracts to rabbits had any influence on the capacity of the liver tissue to reduce oxidized glutathione (GSSG).

Experimental. English hutch rabbits, maintained on a standard diet of cabbage, bran and carrot, were used. The anterior pituitary extract was of the crude saline type described by Ennor (1939), and contained merthiolate in a concentration of 1/1,000. Three subcutaneous injections, each of 5 ml., were given at 12-hourly intervals and the animals were killed 12 hours after the final injection. GSSG was prepared from GSH by oxidation with H_2O_2 after Pirie (1931). Except for some modifications, our technique followed that of Hopkins and Elliott (1931–2). The chief difference in our method was that the extracts contained phosphate buffer which, under the conditions of our experiments, proved to be without effect on the reduction mechanisms.

Variation of the pH from 6·8 to 7·9 was without effect on the reduction mechanisms, but increased acidity (pH 5·6) caused inhibition. On the alkaline side, at pH 8·6, there is also an inhibition. All experiments have been carried out with a M/15 phosphate buffer system at pH 7·6.

Experiments with anterior pituitary extract. The animals treated with anterior pituitary extract were killed by a blow on the head and exsanguinated by division of the carotid artery. In the liver samples the initial GSH content, the GSH content after the experimental period, and the GSH content after the experimental period following the addition of GSSG were determined. GSH determinations were carried out by the method of Woodward and Fry (1932). Two types of extract were used (i) an extract of freshly collected pituitary glands, and (ii) an extract prepared from acetone-dried glands which, prior to drying, had been stored from 3–5 months at 0° C. In all cases sterility tests were carried out on the extracts and it was found that in the presence of merthiolate a sterile condition was maintained for at least 48 hours, after which time the extract was discarded.

The results obtained, using animals subjected to various treatments, are summarized in Table 1.

From the figures shown in normal animals treated with anterior pituitary extract, it is apparent that this has had no effect upon the rate of reduction of added GSSG. On the other hand, as can be seen from columns (a) and (b), there is a definite formation of GSH in animals treated with extract. This indicates that, whilst the excised liver tissue of untreated animals contains no GSSG, this compound does exist in livers of treated animals. Attempts to estimate directly GSSG content were unsatisfactory. The method of Zn reduction and subsequent iodate titration was tried but in common with other workers we found that it was impossible to obtain

[1] This work was carried out under grants from the National Health and Medical Research Council.

TABLE 1.

Increase in GSH in mg. per gm. of liver after 3 hours' incubation at 37° C.

Normal controls.		Injected with anterior pituitary extract.			
		(i) Fresh glands.		(ii) Old glands.	
No GSSG added	50 mg. GSSG added	No GSSG added (a)	50 mg. GSSG added	No GSSG added (b)	50 mg. GSSG added
0 (18)	2·6 (18)	0·4 (15)	2·8 (15)	0·5 (7)	3·2 (7)
Toxaemic animals.		Adrenalectomized animals.		Adrenalectomized + extract.	
0·4 (6)	2·7 (6)	0·0 (6)	2·4 (6)	0·7 (5)	3·3 (5)

Figures in parenthesis indicate number of experiments performed.

good recoveries of added GSSG. In this connection Dohan and Woodward (1939) suggest that Zn is unable to reduce GSSG in a protein filtrate. An electrolytic reduction method described by the above workers was also tried, but gave unreliable results. It was found that if GSSG is placed in the cathode compartment and reduction allowed to proceed, one obtains a recovery in excess of the theoretical. Using a current density of 2·2 milliamperes per sq. cm. and a solution of GSH (50 mg. p.c.) in the cathode compartment, we have found that aliquots withdrawn at 10-minute intervals for iodate titration gave values indicating a progressive rise until at the end of 30 minutes the titre of iodate had increased by 1·0 ml. in excess of the theoretical. In view of these anomalous results, we have not proceeded further with estimations of GSSG, but as an index of its presence have determined GSH derived from the reduction of this compound, using the reduction mechanisms in the liver.

In some of the earlier experiments before the use of merthiolate, contamination of the extract occurred and the animals developed toxic symptoms. In view of these findings, which naturally are not included in our results, it was thought worth while to investigate the effect of toxaemia on reduction of GSSG.

Experiment. A mild diphtheritic toxaemia of 24 hours' duration was produced in rabbits by the subcutaneous injection of 3 M.L.D. of diphtheria toxin. Liver mixtures were prepared and incubated as usual. From results shown in Table 1 the toxaemia appears to produce an increase in GSH in liver samples to which no GSSG was added. This increase, as before, is assumed to denote the presence of GSSG in the freshly excised tissue. No effect on the reduction of added GSSG is apparent.

Experiments were also carried out on bilaterally adrenalectomized rabbits and, as shown in Table 1, the administration of anterior pituitary extract still produced the effects described.

Thermolability of extracts. In an attempt to gain some information as to the fractions producing the described effects on glutathione metabolism we have investigated their ability to withstand heat treatment. Both types of extract were placed in boiling water for approximately one hour and the usual volume of clear filtrate was injected at the customary time intervals. The results of these experiments are shown in Table 2.

TABLE 2.

Increase in GSH in mg. per gm. liver.

Heated fresh gland extract.		Heated dry gland extract.	
No GSSG added.	50 mg. GSSG added.	No GSSG added.	50 mg. GSSG added.
0	2·7	0·1	1·4
0	2·1	0·0	2·6
0	2·5	0·0	2·5
0	1·9	0·0	2·7
		0·0	3·8

Comparison with Table 1 indicates that the fraction responsible for the appearance of GSH during anaerobic incubation of chopped liver (in absence of added GSSG) is thermolabile. Thus experimental results shown in Table 3 are of interest. Here GSH contents of livers are given and also percentage decrease in glutathione in liver extracts shaken in the presence of air at 19° C. for 6 hours. For technique, see Ennor (1939).

TABLE 3.

Acetone-dried gland extract. Untreated.		Fresh gland extract. Untreated.	
Liver GSH mg. p.c.	P.c. decrease of GSH in extract in 6 hours.	Liver GSH mg. p.c.	P.c. decrease of GSH in extract in 6 hours.
300	6	150	3·5
150	8	120	4·0
120	7	200	10·0
128	0	170	12·0
Heated in boiling water for 1 hour.		Heated in boiling water for 1 hour.	
280	25	350	12·0
310	18	295	27·0
275	16	300	10·0

From these results it appears that the factor producing a decreased rate of oxidation of the tripeptide is thermostable.

SUMMARY.

GSSG is present in the livers of normal and adrenalectomized rabbits treated with anterior pituitary extract and suffering from toxaemia.

Rates of reduction of GSSG in livers of normal rabbits, of those receiving anterior pituitary extract and diphtheria toxin and of adrenalectomized rabbits, both treated and untreated, show no significant differences.

Fractions of extracts responsible for (a) production of a diminished GSH content in the liver, and (b) appearance of GSSG are not identical with those producing a diminished rate of oxidation.

At the moment no explanation can be made for the presence of GSSG in the treated animals since the reduction mechanisms, as far as we have been able to study them, appear to be quite unaffected. Indeed, its presence is rather puzzling in view of the fact that the rate of oxidation of the reduced form is slowed. Possibly investigations now being carried out on other oxidation mechanisms in animals receiving similar treatment will throw some light upon these results.

Acknowledgment. The authors wish to thank the Director of the Commonwealth Serum Laboratories for a supply of diphtheria toxin.

REFERENCES.

Dohan, J. S. and Woodward, G. E. (1939): J. biol. Chem., 129, p. 393.
Ennor, A. H. (1939): Austral. J. exp. Biol., 17, p. 173.
Hopkins, F. G. and Elliott, K. A. C. (1931–2): Proc. Roy. Soc., Series B, 109, p. 58.
Pirie, N. W. (1931): Biochem. J., 25, p. 614.
Woodward, G. E. and Fry, E. G. (1932): J. biol. Chem., 97, p. 465.

STUDIES IN THE EPIDEMIOLOGY OF Q FEVER

7. THE BIOLOGY OF *HAEMAPHYSALIS HUMEROSA* WARBURTON AND NUTTALL (ACARINA, IXODIDAE) IN QUEENSLAND

by D. J. W. SMITH

(From the Queensland Department of Health and Home Affairs, Brisbane).

(Accepted for publication 29th January, 1941.)

The rôle of the tick *Haemaphysalis humerosa* Warburton and Nuttall in the epidemiology of Q fever has been discussed in earlier papers of this series in this journal (see bibliography); it is a known vector. In the present paper some details are given of its biology; these observations were made during the course of the other studies mentioned above.

Description and Host List.

The species was originally defined by Warburton and Nuttall (1909), from specimens taken from the bandicoot *Isoodon macrourus* Gould on Barrow Island, N.W. Australia. The same authors (1915) amplified their original description using material from Northern Australia and New South Wales. They did not describe the larva, of which the following details are now given.

Larva (unfed). Body oval 0·6 × 0·5 mm. (without capitulum). Broadest just behind coxae III. Gorged larva 1·3 × 0·75 mm. Scutum broader than long 0·24 × 0·29 mm., cervical grooves well marked, almost attaining the posterior border. Capitulum 0·12 mm. Article 2 of palps approximately equal to article 3. Hypostome with 2/2 files of 5–7 teeth. Well marked spur on coxa I. Coxae II and III elongated, spurs minute.

The following are additional locality and host records.

Queensland: During the Q fever investigations we have collected specimens from *Isoodon torosus* at Palmwoods, Woombye, Nambour, Kiamba and Moreton Island, 1937–40; *Rattus rattus* at Ipswich, XI 1937, and *R. culmorum youngi* at Moreton Island, IX 1938. We have received adults collected by Dr. Heaslip from a bandicoot at Cairns (1939). Through the courtesy of Dr. Roberts of the Animal Health Station, Yeerongpilly, we have examined specimens taken from *Trichosurus vulpecula* at Harrisville, XII 1910, and Springsure; *Eurystomus orientalis* at Maryborough, XII 1911; cattle at Rockhampton, XI 1913, and a horse at Helidon, VII 1933.

There are no records of the tick attacking man, but it has been found to do so when tested experimentally in the laboratory.

Life Cycle.

(i) *Larvae.* *H. humerosa* is a three-host tick, dropping off the host after engorgement at each successive stage of the life cycle.

Larval ticks hatched in the laboratory were fed upon guinea-pigs at intervals ranging from approximately 10 to 80 days after hatching. They generally became attached to the host within 12 hours, and the majority of them were fully engorged within 36 to 48 hours thereafter. During cool weather, ticks were tardy in beginning to feed and the majority of several batches tested remained in the enclosing capsule for 2–4 days before attaching to the host.

Upon bandicoots captured in the bush, larval and nymphal ticks were most frequently found attached to the feet, hindquarters and tail.

The time taken by engorged larvae, after dropping from the host, to moult to the nymphal stage varied from 14 days at a mean monthly temperature of 70·2° F. to 33 days at 58·6° F.

(ii) *Nymphae.* Nymphal ticks were placed upon guinea-pigs at intervals ranging from 4 to 42 days after ecdysis. The majority of them attached within 12–24 hours, falling from the host in the engorged state after a further 48–72 hours. The minimum length of the nymphal stadium was found to be 14 days, at a mean monthly temperature (at Brisbane) of 70·2° F. At a mean monthly temperature of 58·6° F. it was 39 days.

(iii) *Adults.* Adult ticks were placed upon guinea-pigs at intervals of 5–48 days after ecdysis. The feeding of male ticks extended over a long period; ticks were removed when still feeding, 4 to 5 weeks after being placed on the host. They frequently changed their site of attachment. Bandicoots captured in the bush and brought into the laboratory always carried many more males than females; the males remain on hosts which the females have abandoned. The females were most commonly found feeding in the region of the neck and shoulders. Female

ticks fed upon guinea-pigs usually engorged and dropped from the host after 8–14 days, though occasionally individuals engorged only slowly over periods up to 26 days.

Coupling occurred only on the host. It was commonly observed from two to four days after feeding had commenced. In the absence of males, females remained attached to the host, but engorgement was not completed. Three such partially engorged ticks were removed from an animal after 12 days, and 8 days later placed upon a second host. After 12 days attachment to the latter animal without visibly increasing in size they were removed, to die without ovipositing in the succeeding week.

Observations made on the oviposition of 20 ticks are summarized in Table 1. Ticks reared in the laboratory upon guinea-pigs neither attained quite the same size during engorgement as those fed upon the bandicoot, nor did they lay as many eggs.

TABLE 1.

Observations relating to oviposition.

Number of ticks used.	Pre-oviposition period.	Oviposition period.	Number of eggs laid. Bandicoot.	Guinea-pig.	Survived after oviposition ceased.
20	Mean 5·7 days Range 5–15 days	Mean 13·7 days Range 10–20 days	Mean 485 Range 200–1,000	Mean 138 Range 50–260	Mean 14·5 days Range 2–26 days

(iv) *Eggs.* Observations on the rate of development of the eggs, based on 12 egg batches, showed that the first larvae emerged in 21 days at a mean monthly temperature of 64·5° F.; they took 65 days during two winter months when the mean monthly temperatures were 60·2° F. and 58·6° F. respectively.

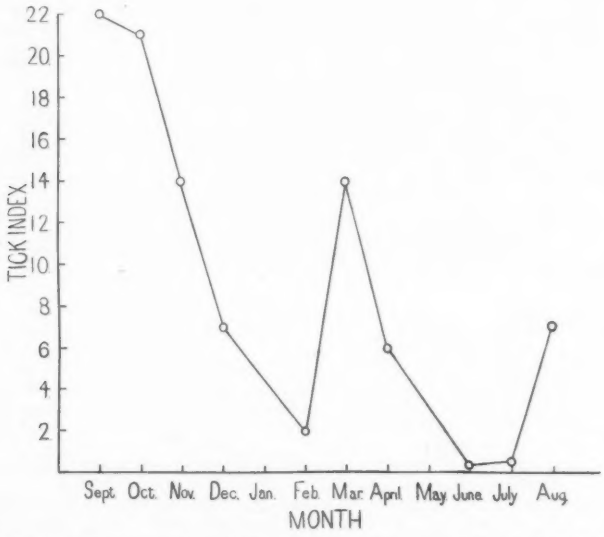


Fig. 1. Showing the seasonal variation in the average number of adult ticks found upon bandicoots, in an examination of 113 animals from Moreton Island during the period of September, 1938–August, 1939.

(v) *Duration of life cycle.* Larval ticks were in one instance observed to live at least 72 days. Several nymphs remained alive for 115 days, but all were dead 9 days later. Ten males and five female ticks lived at least 73 days.

The time required to complete the life cycle, based on the foregoing observations, is summarized in Table 2. Under favourable conditions, such as obtain on Moreton Island and along the coastal scrub areas of Southern Queensland, it is probable that at least two generations occur annually.

TABLE 2.

Time required to complete life-cycle.

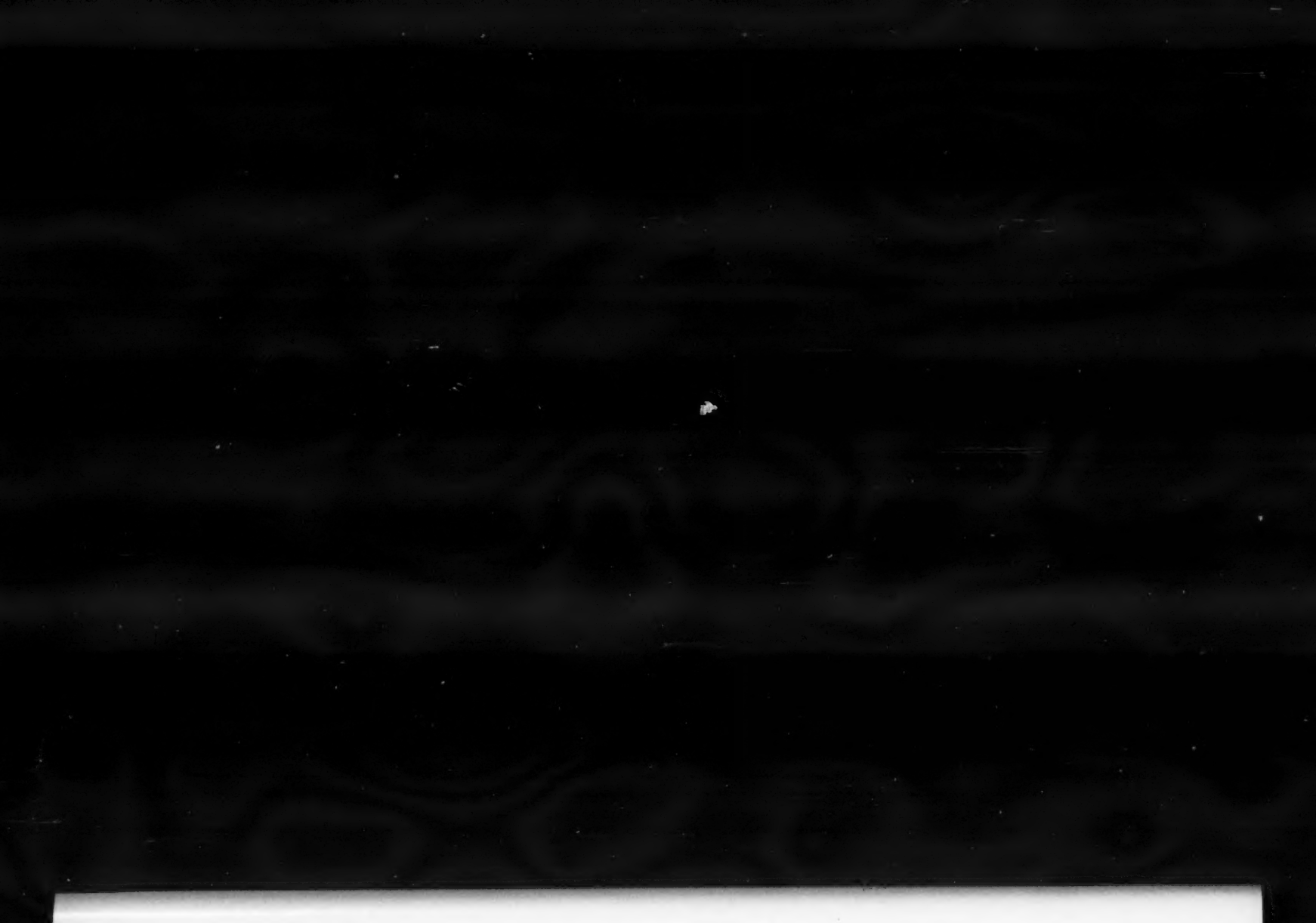
	Days required. Shortest period.	Longest period.
Fecundated ♀ abandons the host, begins to oviposit	5	15
Larvae hatch out after	21	65
Larvae harden and wait for host No. 1	7	72
Larvae remain on host	2	4
Nymphs issue from larval skin	14	33
Nymphs harden and wait to attach host No. 2	7	115
Nymphs remain on host No. 2	3	5
Adults issue from nymphal skin	14	39
Adults harden and await host No. 3	7	73
Adults remain on host	8	26
Totals	88 days	447 days

(vi) *Seasonal incidence.* The results of the examination of nearly 250 bandicoots during the period July, 1937–July, 1940 are as follows. Adult ticks, male and female, were found during each month throughout the year; a well marked seasonal variation in their number was noted (Fig. 1). Larval and nymphal ticks were not found during the hot months (December–February). They were present throughout the rest of the year, and like the adults, exhibited a marked increase in numbers during early spring and autumn. As the majority of animals were examined at the laboratory, usually two or more days after capture in the bush, the absence of immature ticks during the summer months may have been due to their having abandoned the host prior to examination.

SUMMARY.

An account is given of the occurrence and life cycle of the tick, *Haemaphysalis humerosa*, a known vector of Q fever.

Acknowledgments. I am indebted to Sir Raphael Cilento, Director-General of Health and Medical Services, Queensland, for permission to publish this paper, also to Dr. F. H. S. Roberts of the Animal Health Station, Yeerongpilly, for confirming the identity of the tick and for the loan of specimens from his collection.

REFERENCES.

Freeman, M., Derrick, E. H., Brown, H. E., Smith, D. J. W. and Johnson, D. W. (1940): Austral. J. exp. Biol., 18, p. 193.
Nuttall, G. H. F. and Warburton, C. (1915): A Monograph of the Ixodoidea, Part 3, p. 496.
Smith, D. J. W. and Derrick, E. H. (1940): Austral. J. exp. Biol., 18, p. 1.
Smith, D. J. W. (1940): *Ibid.*, 18, p. 103.
Warburton, C. and Nuttall, G. H. F. (1909): Parasitology, 2, p. 60.